

Exploring the antidiabetic potential of protein hydrolysates derived from underutilized marine sources

A thesis presented for the degree of

Doctor of Philosophy

In the

School of Biomedical Sciences

Diabetes Research Group

Faculty of Life & Health Sciences



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July 2018

(I can confirm the word count for this thesis is less than 100,000 words)

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Acknowledgements

I would like to acknowledge the outstanding support and guidance from Professor O'Harte during this PhD project, including all who have giving me the time and independence to develop as a researcher such as Dr Allsopp, Dr Parthsarathy, Dr McSorley, Professor McKillop and Professor Flatt. I have spent the past few years, without question, enjoying my time within the Diabetes Research Group. I would like to thank each staff member including the most important aspect of the group, the students, old and new, for making the time fun, exciting and challenging. A special thanks to Michael, Neil, Andrew, Ryan and Shruti for being fantastic people.

To Vanessa, Kiara and Darragh, each of you have brought me joy, happiness, support, kindness and understanding throughout this journey of what must seem like one long uphill struggle of late nights, weekend BBRU visits and at times the grumpiness that accompanies both. Without each of you, this would not have been worth the effort.

Dad, the work within is dedicated to you

We do not cry because we lost you,

We smile because we had you.

Summary

In Type-2 diabetes, the classic biguanide and sulphonylurea drugs remain first line treatments for alleviating chronic insulin resistance and hyperglycaemia. However, newly emerging DPP-4 enzyme inhibitors or long acting hormone-based therapeutics provide second line alternatives. The discovery of novel peptide therapeutics for the treatment of diabetes and obesity offers simultaneously targeting of multiple molecular pathways. Peptide-based therapies provide highly specific mechanisms for controlling multiple aspects of glucose dysregulation including improving post-prandial glycaemia, satiety and gastric emptying. Dietary proteins must first be hydrolysed to unlock their bioactive peptide components. Investigating the oral efficacy of marine protein hydrolysates was a key aim of this research. Initial screening, using BRIN-BD11, GLUTag and STC-1 cell lines, enabled bioactivities to be identified from a variety of biological protein sources. Generally, hydrolysates generated using multiple proteolytic enzymes, provided a more effective strategy for enhancing insulin, GLP-1 and GIP secretion from cultured cells. The bioactivity blue whiting, boarfish, salmon and *Palmaria palmata* derived peptides varied significantly, interestingly, even when identical enzyme hydrolysis methods were used. Hydrolysates administered orally at 50-150 mg/kg/bw led to an improvement in post-prandial glycaemia and insulinotropic responses in normal mice, but unfortunately in some instances were less effective at reducing food intake and promoting satiety. Long-term oral administration of *Palmaria palmata*, boarfish and salmon skin hydrolysates resulted in improved non-fasting glucose, plasma lipids and terminal glycated haemoglobin in *ob/ob* or Streptozotocin diabetes induced mouse models. Finally, within a double blind placebo controlled crossover trial, glucose tolerance in healthy individuals had resulted in no improvement in post-prandial glucose excursion using 3.5g of boarfish protein hydrolysate, of which had previously shown the most promising *in vitro* and *in vivo* results. Future study will need to assess the quantity of protein hydrolysate required for humans aiming to present the same positive effects seen within the cell and animal study models.

Abbreviations

AAC	Area above the curve
AUC	Area under the curve
BMI	Body mass index
BPH	Boarfish protein hydrolysate
BWPH	Blue whiting protein hydrolysate
CLAMS	Comprehensive Laboratory Animal monitoring Systems
DEXA	Dual-energy X-ray absorbance
Da	Dalton
DPP-4	Dipeptidyl peptidase 4
FBS	Foetal Bovine Serum
g	Gram(s)
GLP-1	Glucagon-like peptide 1
GTT	Glucose tolerance test
h	Hour(s)
HbA1c	Glycated haemoglobin A1c
HBSS	Hanks buffered saline solution
HCl	Hydrochloric acid
IP	Intraperitoneal
Kg	Kilograms
KRBB	Krebs ringer bicarbonate buffer
L	Litre
LDL	Low-density lipoprotein
M	Molar
MCi	MilliCurie
mg	Milligram
min	Minute
ml	Millilitre
mmol	Millimolar

n	Number of observations
NIH	National institutes of health
OGTT	Oral glucose tolerance test
P	probability
PBS	Phosphate buffered saline
PPPH	Palmaria palmata protein hydrolysate
RIA	Radioimmunoassay
RP-HPLC	Reverse phase high performance liquid chromatography
sec	Second
SEM	Standard error of the mean
SGID	Simulated gastrointestinal digestion
SGPH	Salmon gelatine protein hydrolysate
STPH	Salmon trimmings protein hydrolysate
T	Time
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TFA	Trifluoroacetic acid
μg	Microgram
μl	Microlitre
μM	Micromole

Declaration

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Chapter 1

Marine biopeptides and their therapeutic applications

Background & Aims

Marine protein, hydrolysates and derived small molecular weight peptides, have in recent times been investigated for their pleotropic effects upon multiple therapeutic areas. This chapter aims to highlight current literature, legislative and therapeutic outcomes. To date, the key message for protein intake are still convoluted in regards to recommended intakes for health promotion or disease prevention, however with this noted, researchers globally are continuing to report anti-cancer, anti-obesogenic or glucoregulatory effects of novel proteins and their subsequent peptide hydrolysates using well-established models of investigation. The role which proteins are involved in above nutrition and metabolism, is still a topic of debate due to the unknowns surrounding the effect of liberated peptides after oral ingestion. For this reason, this chapter investigates pre-digested proteins or peptides identified after hydrolysis, and their effect on health.

1 Introduction

The identification of bioactive compounds has, in more recent times, shifted from terrestrial environments to rivers, coasts and oceanic depths. The earth, while covered by 70% oceans supports merely 15% of all living organism (Grosberg *et al*, 2012). Diverse marine biological organisms have adapted, responded to aggressive climate change and thrived in environments foreign to terrestrial organisms. The ability to adapt rapidly to temperature, salinity, biological and chemical changes has pioneered the search for unique molecular compounds contained within living marine organisms. The oceans are considered one of the most abundant sources of bioactives (Lordon *et al*, 2012) with current environmental efforts set in place to increase oceanic sustainability, diminish industrial pollution and promote a return of the diverse pelagic fish stock and dwindling coral reefs (Pauly *et al*, 2005). With this being noted, the exploitation of marine organisms for bioactive molecules is underway and the race for identifying the next notable biologically active and therapeutic compounds taking lead.

The current costs associated with marine exploration the investigation of bioactive marine molecules requires rigorous characterisation and considerable effort. Unfortunately, in most cases the true potential of bioactive compounds are unknown (Nasri *et al*, 2014). Never-the-

less this does not hinder the current global approach to discovering bioactive molecules and their specific application. Tailored approaches using modern problematic healthcare areas such as infection and immunity (Cheung *et al*, 2015) diabetes and obesity (Lauritano & Ianora, 2016), neurology (Grosso *et al*, 2014), cancer and tumorigenesis (Xing, *et al*, 2017; Suarez-Jimenez *et al*, 2012) and cardiovascular disease (Pangestuti & Kim, 2017) are rapidly growing. Marine life is vast with molecules of which many are yet to be fully understood. The global market for nutraceuticals has increased year on year with annual revenues of US\$ 200 billion. Current production of nutraceuticals relies heavily on extraction from source followed by identification and purification of the compound and ultimately screening of its bioactivity. Unfortunately research compounds identified begin with a low cost-per-tonne ratio, however, improving a compounds bioactivity can significantly increase market value (Cudennec *et al*, 2012; Martins *et al*, 2014). Recently. molecules of low cost and in current high demand are polysaccharides, polyunsaturated fatty acids (PUFAs),, proteins, enzymes, minerals and vitamins, with this noted, large scale removal of protein based compounds carries increased cost association versus some of the more traditional extraction methods(Grosso *et al*, 2015).

Assessment of bioactivity is firstly screened at lab scale, this is noted with marine polysaccharides, fatty acids, protein and subsequent hydrolysis to yield smaller novel research molecules. Bioactive peptides are of particular interest and contained within larger protein molecules and as such need liberated to exert biological activity. Protein extracts are initially extracted using acidic or alkaline techniques (Tsugita & Scheffler, 1982), followed by further sequential hydrolysis generation via a controllable and adjustable addition of proteolytic enzymes (Polanco-Lugo *et al*, 2014; Martínez-Maqueda, *et al*, 2013). Peptide-based nutraceutical or drug design is a specialised endeavour aimed as serving as potent pharmacophores. Natural peptide approaches are favoured due to specificity and high tolerance profiles (Fosgerau & Hoffmann, 2015). Peptide based therapy approaches is a diverse venture with peptides serving as multifunctional signalling molecules or conversely very specific cell binding molecules of which can be cell or receptor specific targeting molecules, as such promising cell penetrating, uptake and apoptotic properties have been reported using *in vitro* approaches (Boohaker *et al*, 2012). The oral delivery of nitrogen

containing molecules has its limitations, including rapid degradation via endogenous intestinal and pancreatic proteolytic hormones.

Both terrestrial and oceanic environments provide a variety of peptides that are expressed in most living species. This has created evolutionary optimised response of peptides to bind to cellular receptors with agonistic or antagonistic mechanisms. However, the hydrolysis of protein can generate an abundance of less common or completely uncharacterised peptides of which signalling mechanisms are to be established (Harnedy *et al*, 2015). Naturally occurring proteins and peptides consist of amino acids typically in their L-isoform configuration. Studies involving naturally occurring peptides with D-amino acids have reported resistance to endogenous degradation (Bai *et al*, 2009).

Using several proteolytic enzymes may improve sequential hydrolysis outcome. Hydrolysis of marine protein using aqueous solution coupled with proteases solution can yield a magnitude of small molecular weight peptides (SMWPs). The overall degree of hydrolysis of intact protein has several outcomes, a broad pool of fragmented peptides dependent on hydrolysis time followed by separation based on fragment size (Hong *et al*, 2012), mass (Gundry *et al*, 2010), charge (Burgi & Smith, 2001) or the most commonly used method of hydrophobicity separation (Conlon, 2007). This novel approach to generating bioactive compounds from marine proteins and their application focused towards current therapeutic potential will be the focus of this review.

1.2 Sources of marine protein

With population growth, reduction of arable farming land and fresh water shortages, macronutrient sources are becoming ever challenging. There is a global race to provide sustainable high quality protein sources. Sustainability efforts and reduction or re-use of marine waste has opened a new revenue stream for fisheries (Senevirathne & Kim, 2012). Fresh approaches has pioneered government backed research to tackle this area and identify solutions of usability for all aspects of marine nutrition (EU Common Fisheries Policy, 2014 – 2020).

Hauls of unwanted ichthyoid species are now landed and stored long-term. This gives fisheries the opportunity to wholesale larger quantities of non-marketplace fish that yield high quality protein among other constituents. Fish waste, processing by-products or non-marketable species are often used as fertilizer as well as domestic and livestock animal feed. The extraction of potentially upgradable functional food components has recently shifted that outlook (Cudennec *et al*, 2012). Successfully harvesting marine protein rely firstly on availability of natural resources of which coastal areas are affected by potentially faltering weather conditions and seasonality. Long-term economical and exploitable sources of macroalga are being developed with coastal expansion and sustainable livelihoods in mind (Rebours *et al*, 2014). Coastal areas are now one of the most exploitable areas of marine harvesting.

Macroalga production worldwide has a revenue stream estimated to exceed US\$ 10 billion. Seaweeds are consumed by coastal communities across the globe daily and serves as a habitual addition to many diets. The demand for seaweed is increasing, especially for seaweed protein for ingredients (Černá, 2011). Asian countries dominate macroalga harvesting and production with 80% consumed for nutrition with remaining sold for animal feed, fertilizers, pharmaceutical and biotechnological applications. There are over 250 species of seaweed worldwide, 150 currently classed as edible and many yet to be fully characterised. The nitrogen and protein content has become the focus of applied research for seaweed in more recent times (Harnedy *et al*, 2014). Protein content of seaweed varies between species with brown (Phaeophyceae) seaweed having the lowest mean protein content of approximately 10% (DW) with red (Rhodophyta) and green (chlorophyta) seaweed averaging 13% and 15% total protein (DW), respectively (Černá, 2011). The harvesting period marine of macroalga plays an important role in total protein content with protein levels at their highest in May and subsequently decline over the summer months (Galland-Irmouli *et al*, 1999). The explanation for lower protein content in the warmer months is factored on decreased water nitrogen content accompanied by nitrogen assimilation and energy consumption for growth in warmer climates (Kumar *et al*, 2014). *Palmaria palmata* (Dulse) a red seaweed native to northern coast of the Atlantic and Pacific oceans remarkably reported to contain as high as 30-45% protein (Galland-Irmouli *et al*, 1999). Other emerging sources of biomining and protein extraction are that of marine fungi and bacteria, shellfish, crustaceans, and a relatively

new area utilizing fish industry processing discards and fish waste water. These sources are now of considerable importance and potential sources of biologically active compounds and source of nutrition.

1.3 Marine protein hydrolysates as potential therapeutics for type 2 diabetes

Diabetes mellitus is a chronic degenerative disease with high rates of complications that are now impacting all areas of specialised healthcare, and as such carries a high morbidity and mortality rate (ADA, 2010). Type 2 diabetes mellitus (T2DM) is characterised by hyperglycaemia, abnormal insulin levels, lack of insulin secretion and functioning and eventually pancreatic beta-cell failure if uncontrolled (Kahn, 1994). Disease aetiology closely shadows lifestyle factors such as, excessive nutritional intake and lack of physical activity. In obese individuals there is a marked increase in hypertension, cardiovascular disease, non-alcoholic fatty liver disease (NAFLD), and dyslipidaemia and increase risk of certain cancers. Initial treatment methods are being rapidly surpassed for alternative options; however, the consensus remains, majority of cases can be completely evaded by the adoption of a healthier lifestyle (Chong *et al*, 2017).

Chronic hyperglycaemia favours intensive pharmacological treatment approaches. As such the use of metformin (Glucophage), a hepatic glucose liberation inhibitor is being combined with more exotic oral glucose lowering agents or injectable degradation resistant peptide molecules (Marshall, 2017). Oral hypoglycaemic agents such as the family of gliptins (sitagliptin, vildagliptin, saxagliptin *et al*), inhibit the activity of an endogenous enzyme dipeptidyl peptidase IV (DPP4) by binding rendering the enzyme redundant (Scheen, 2010). Endogenous DPP4, a type II transmembrane glycoprotein cleaves highly active and potent postprandial incretin peptide hormones that play vital roles in insulin secretion, specifically glucagon like peptide 1 (GLP-1) and glucose-dependant insulinotropic polypeptide (GIP) (Matteucci & Giampietro, 2009). Sustained or Increase activity of the incretin hormones have glucoregulatory activity including decreased gastric emptying, increased glucose dependant insulin secretion, lower glucagon production and increase β -cell proliferation with decrease apoptosis (Holst *et al*, 2009). As such pharmaceutical companies have identified a need for longer acting, degradation resistant molecules that act upon endogenous hormone pathways.

An identified peptide molecule from a Gila monster (*Heloderma suspectum*) venom is pharmaceutically available under the name exendin-4 (Byetta) and used in the treatment of hyperglycaemia in patients with type 2 diabetes (DeFronzo *et al*, 2005). The peptide agonist binds to the glucagon-like-peptide 1 receptor (GLP-1r) and facilitates the secretion of insulin from the pancreatic β -cells, while sharing only 50% similarity in amino acid sequence and presents double the half-life over the native GLP-1 hormone. This has promoted researchers to further investigate the proteomic approach of improving debilitating conditions such as type 2 diabetes (López-Villar *et al*, 2015). Pharmaceutical approaches to treating disease states have the added drawback of accompanied side effects, in particular the cardiovascular issues that currently surround the use of DPP4 inhibitors (Sheen, 2013). It is postulated that oral bioactives and nutraceuticals carry fewer risks via improved tolerance (McCarty, 2005).

Anti-diabetic molecule screening approaches of protein hydrolysates have shifted from large polypeptides to much shorter peptide chain length (Xia *et al*, 2017). Recent approaches using marine protein hydrolysates displayed markedly reduced fasting glucose concentration using chum salmon (*Oncorhynchus kern*) in a rat model of type 2 diabetes (Zhu *et al*, 2010). Among the proposed mechanisms for reported antidiabetic potential lays the ability of protein hydrolysates to inhibit promoters of T2DM which may lead to disease progression. Biopeptides derived from marine hydrolysates have been recently investigated for their DPP4 inhibitory activity (Huang *et al*, 2012; Wang *et al*, 2015). Extraction of small molecular weight peptides from *Palmaria palmata* protein hydrolysate displayed *in vitro* DPP4 enzyme inhibition (IC_{50}) at 43.4 (ILAP), 53.67 (LLAP) and 159.37 (MAGVDHI) $\mu\text{mol/L}$ respectively (Harnedy *et al*, 2015). A recent study investigating parameters of circulating glucose dysregulation in *db/db* rats reported an improvement in excretion products such as glucose and urea content in urine within a group given oral polypeptides from sea cucumber (Li *et al*, 2017). Glucose and lipid regulatory activity was seen in a group of 100 diabetic cohorts after treatment with marine collagen peptides (Zhu *et al*, 2010). Daily oral administration resulted in significantly lower fasting blood glucose levels, reduction in glycated haemoglobin (HbA_{1c}), fasting blood insulin, improved lipid parameters, notably reduced cholesterol, triglycerides and elevated high density lipoprotein (HDL) following treatment for 1.5 or 3 months versus control group, respectively.

Bioactivity and mechanism of action is one of the more challenging aspects of molecule discovery. Oligopeptides from marine salmon skin (OMSS) administered daily (4 g/kg/d) to diabetic rats for 4 weeks resulted in significantly reduced fasting blood glucose, lower circulating serum levels of TNF α and IFN γ and diabetes oxidative stress marker malondialdehyde (MDA). Furthermore, resulting antioxidant enzymes measured, specifically SOD and GSH were increased (Zhu *et al*, 2010). The improvement in diabetes status *in vivo* often involves the measurement of oxidative stress markers, whether this is a direct or indirect effect of disease status or marine protein hydrolysates ingestion is yet to be established. Marine collagen peptides (MCP) administered to a rodent model of type 2 diabetes reported that 9 g/kg/day significantly improved glucose, insulin and insulin sensitivity and subsequently improved the insulin sensitivity index comparative to control, respectively. Lower doses of MCP, 4.5 and 2.25 g/kg/d significantly improved liver steatosis (Zhu *et al*, 2017). A similar dose of >4.5 g/kg/d of MCP given to rats partially ameliorated β -cell apoptosis and apoptosis markers versus a glucotoxic solution alone when studied *ex-vivo* (Zhu *et al*, 2017). The results demonstrated here have displayed the potential dietary addition of marine protein hydrolysates to partially alleviate the negative outcomes of glucoregulatory issues using *in vitro*, *in vivo* and human models of disease study. Implementing bioactive marine peptides as well as the classical and pharmaceutical approaches for the treatment of type 2 diabetes may have additive benefits in reducing complications associated with the disease.

1.4 Anti-cancer effects of marine protein hydrolysates

The impact of dietary choices and nutritional status are the most important determinants of chronic disease controlling for genetic predisposition. The anticancer and cancer preventative potential of marine bioactives has been a focus of great interest over the past several decades and as such has focuses on their antiproliferative, antioxidant and antimicrotubule actions (Suarez-Jimenez *et al*, 2012). Countries that habitually incorporate marine nutrition into their diet have reported positive health outcomes. Asian countries with high intake of seafood have incidence rates lowering in particular cancers, such as breast, prostate, lung and colorectal compared to North America and Europe (Key *et al*, 2004).

Identifying the observed trend associated with nutritional intake of seafood and the decrease cancer rates has led to extensive research aimed at identifying the beneficial compounds present within. Interestingly some local delicacies are also used as ingredients in folk medicine in certain areas of Asia (Sho, 2001). Previously interest in consumption patterns of fish and fish products, particularly cod and cod liver oil, Eel, of which is a staple of many Asian diets and bivalves such as mussels, oysters and clams. Fish consumption is associated with health benefits with more recent approaches highlighting the anticancer effect of marine protein, hydrolysates and peptides (Suarez-Jimenez *et al*, 2012). Fish oils, fish meal and domestic and livestock applications are shifting attention now towards identification of bioactives from marine sources such as fish scales, muscle and organ protein of which as a marine product, is only beginning to be appreciated as effective therapeutics (Haefner, 2003). Investigations of marine protein hydrolysates and anticancer activity are however, currently scarce. Studies involve the use of cancer cell lines and report growth performance or antiproliferative activity when incubated with fish protein hydrolysates. There is however a major limitation, tracking the direct effect of marine bioactives, either protein hydrolysates or biopeptides and their therapeutic effect in humans is a difficult endeavour and currently relies heavily on self-reported retrospective data of which tracks prevalence rather than causation and prevention (Schüz *et al*, 2013). However, as such the use of human cancer cell lines and small rodent cancer studies may give insight into the anticancer or therapeutic benefits of marine bioactives.

A study investigating 18 enzymatically digested hydrolysates from various species of fish had a spread of peptides from di- to tripeptides to polypeptides of 7 kDa in mass. Incubation with several FPH, specifically blue whiting, which contained 96% peptide content resulted in up to 30% reduction in cellular proliferation in MDA-MD-231 cancer cells and up to 26% proliferation inhibition in MCF-7 human breast cancer cells (Picot *et al*, 2006). Similar reduction was seen when incubated with cod, plaice and salmon protein hydrolysates. The observation was concluded that direct cytotoxicity was present when specific peptides were exerting an anticancer effect on the cell lines. The mechanism of anti-cancer investigation using ultrafiltrated roe hydrolysates (URH) extracted from giant grouper (*Epinephelus lanceolatus*) promoted a dose-response reduced cell viability of two oral cancer cell lines using an ATP based activity assay in Ca9-22 and CAL 27 cell lines, respectively (Yang *et al*,

2016). The cellular proliferation reduction and promotion of apoptosis using flow cytometry to determine apoptotic changes reported sub G1 accumulation of cells. The apoptotic effect was deemed to arise from induced oxidative stress in Ca9-22 cells when incubated with ultrafiltrated roe hydrolysates. The bioactivity of marine protein hydrolysates to inhibit cellular cancer proliferation or undergo controlled cell death has been noted, the ability of MPH to inhibit the metastatic potential of a malignant mesenchymal tumour was described using seahorse-derived (*Hippocampus kuda*) peptides (SP) with a similar amino acid sequence of α -enolase, a glycolytic enzyme expressed in most tissues. Using fibrosarcoma cells (HT1080) the seahorse peptide decreased interaction of α -enolase and plasminogen, both promoters of metastatic potential of cancer cells. The investigation took place by mixing the SP, α -enolase and plasminogen together prior to incubation with the HT1080 cell line. The decrease in cellular invasion migration over 48 h using the matrigel invasion assay and HT1080 cells was due to the decreased interaction of α -enolase and plasminogen. Since plasminogen and α -enolase interaction are involved in plasminogen activation factors, this signalling was inhibited by the use of sea horse-derived peptides (Kim *et al*, 2014). The anti-cancer activity of marine protein hydrolysates and marine bioactive peptides have relied heavily on their antioxidant capacity of which is a widely reported trait of small molecular weight peptides (Sheih *et al*, 2009) / (Kumar *et al*, 2012). Purified cyclic peptides from marine sponges have been reported antioxidant and cytotoxic activity on several human cancer cell lines such as MCF-7, HeLa, and HepG2 (Zhan *et al*, 2014). However the anti-cancer activity of orally ingested marine protein hydrolysate is more or less unknown with long term intake of marine bioactives from intact sources favoured over extracted bioactives. This leaves the true potential of bioactive marine peptides and long-term intake and efficacy an increasingly difficult outcome to measure.

1.5 Anti-oxidant activity of marine protein hydrolysates

Many human physiological complications have been related with considerable evidence to that of oxidative stress (Uttara *et al*, 2009)/(Hybertson *et al*, 2011). As the body's natural anti-oxidant state is overwhelmed this allows an oxidative state to arise. The body is constantly fighting to maintain oxidative equilibrium, However, several disease states can alter this homeostasis. The oxygen we breathe has undoubtedly obvious benefits to human

physiology however it similarly carries potentially damaging side effects for biological systems. An evolutionary system is in place to instinctively adapt to a rise in oxidative stress and successful attempts are made in counterbalancing this effect (Sies, 1997). Initial minor disturbances in oxidative stress may not have any metabolic consequences due to homeostatic adaptations, however chronically elevated oxidative state may lead to irreparable damage and eventual cell death. This damage arises from reactive oxygen species in two major forms, free radicals and their non-radical substrates. Free radicals are molecules defined as having one or more unpaired electron and incomplete outer shell that confers their highly reactive biological state (Pham-Huy *et al*, 2008). Biological states that are imperative or important to life inadvertently generate reactive oxygen species, such as leakages in the electron transport chain, cytochrome P450 and NADPH oxidase. Other sources of oxidative stress are drug abuse, toxins and smoking.

Dietary antioxidants such as ascorbic acid in the form of vitamin C or vitamin E, in the form of γ -tocopherol, are extremely potent reactive oxygen species quenchers, and as such have imperative dietary roles in reducing oxidative stress (Lobo *et al*, 2010). Target mechanisms of antioxidants are to mop up the molecules of high reactivity by donating an electron to reduce reactivity or binding irreversibly to inhibit duration of activity. The collective term for superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($\cdot OH$), singlet oxygen (1O_2) and perhydroxyl radical ($HO_2\cdot$) is that of reactive oxygen species, and as such are a current research target for molecules that can inhibit their damaging capacity or reduce the subsequent damage to macromolecules such as lipids, protein and carbohydrates. This elevated oxidative state can also result in DNA-protein cross links, double strand breaks, base damage, and protein fragmentation (Ray *et al*, 2012). The first line of innate defence against RoS is conversion of the more potent molecules to ones of lesser damaging effect. This is seen via the enzyme driven interaction with superoxide ($O_2^{\cdot-}$) with superoxide dismutase (SOD) which yields H_2O_2 a lesser reactive molecule of which on its own cannot damage macromolecules. The molecule can however cross cellular membranes and produce the highly reactive hydroxyl radical ($\cdot OH$). On a nutritional level, oxidation causes many problems for the food industry particularly reduction in shelf life, loss of wanted sensory characteristics and promotion of unwanted as well as production of toxic compounds of which are dangerous for health and the consumer. The food industry has an arsenal of synthetic antioxidants which

are widely used to inhibit the retardation of foodstuffs and reduce lipid oxidation which yield rancid smells (Augustyniak *et al*, 2010). As such there has been a global interest in gathering information on natural antioxidants that may be used in favour over synthetic antioxidants which may also present potential to improve health and improve the food we eat.

Marine research has no shortages of antioxidant compounds that have exploded the area of marine protein hydrolysate research and identified peptide compounds (Sheih *et al*, 2009)/(Kumar *et al*, 2012)/(Kang *et al*, 2012). Low molecular weight proteins that have undergone enzymatic hydrolysis to yield peptides and glycoproteins obtained from marine sources have been screened for their antioxidant effect, this is now considered an attractive way of liberating bioactive molecules without impairing their nutritional value. A study using ultrafiltration methods to combine peptides generated from scalloped hammerhead cartilage hydrolysate (SHCH) after various methods of proteolytic degradation concluded that protein hydrolysates with lower molecular weight showed higher hydroxyl radical ($\cdot\text{OH}$) scavenging activity *in vitro* (Li *et al*, 2017). As hydrolysate concentration increased subsequent antioxidant activity showed linear correlation. Further daily assessment over 7 days had shown a dose-dependent decrease in lipid peroxidation with one SHCH fraction showing similar antioxidant activity to the synthetic antioxidant Butylated hydroxytoluene (BHT). A similar approach was seen in the purification of antioxidant peptides from salmon by-products and muscle proteins that were subjected to proteolytic degradation using bacterial extracellular proteases from *Pseudoalteromonas sp.* rather than conventional mass production proteases (Wu *et al*, 2017). Bacterial extracellular proteases were cultured *in vitro* and activity assessed at laboratory scale prior to the protein catabolism and subsequent generation of various salmon protein hydrolysate for screening. Results showed that antioxidant activity was greater from salmon by-product than that of salmon collagen hydrolysates generated. Comparing antioxidant peptide sourced from marine muscle protein displayed an antioxidant activity (DPPH) IC_{50} value of 0.51 mg/ml when hydrolysate was generated using cultured bacterial extracellular proteases mentioned versus gastrointestinal proteases (0.60 mg/ml), neutral serine protease (0.60 mg/ml), trypsin (1.40 mg/ml), pepsin and alcalase combination (1.35 mg/ml) and papain (3.06 mg/ml), respectively. The study demonstrated that antioxidant activity may be limited by the hydrolysate process and which enzyme involved but also the choice of starting raw protein material plays an intrinsic role in

antioxidant outcome. Protein hydrolysates and identified peptides from Croceine Croaker (*Pseudosciaena crocea*) were investigated for their antioxidant capacity (Zhao *et al*, 2016). Hydrolysates were generated using alcalase, papain, pepsin and trypsin, respectively. An identified hydrolysate fraction was used in several scavenging activity assays and DNA damage protective effect assessed. Furthermore the anti-fatigue effect was assessed in prolonged exhaustive swimming time in mice. Results concluded that while the identified protein fraction with bioactivity in scavenging assays was positive in reducing reactive oxidative species activity, particularly as concentration of hydrolysate increased, the study highlight was improved protective activity against free radical induced DNA damage and improvement in exhaustive swimming time relating to an anti-fatigue effect of marine protein hydrolysates extracted and fractioned from Croceine Croaker swim bladder.

Similar exhaustive swimming duration and anti-fatigue effect was assessed using Wistar rats (Huang *et al*, 2015). Rats were separated into groups (n=10) and assigned either dH₂O only, dH₂O and glutathione or dH₂O and different concentrations of Hairtail protein hydrolysate (HPH) ranging from 50 to 200 mg/kg bodyweight with or without Fe chelate. Assigned treatments were administered orally via gavage for 20 days, prior to experimentation assigned treatment was given 1 hour before. Results displayed that rats given HPH alone or Fe chelate alone did not perform as well as animals given HPH/Fe together. An increase of 60% in exhaustive swimming time was pronounced in HPH/Fe (200/12 kg/bw/day) versus negative control (dH₂O) and 20% increase versus positive control group (glutathione (100 mg/kg·bw)), respectively. Furthermore, enhancement of GSH-Px activity and significantly lower blood lactic acid were evident in HPH/Fe group versus negative control group (dH₂O). The study failed to identify the bioactive component producing the anti-fatigue effect however future sports related performance enhancing through supplementation is reasonable. Many studies have reported the intake of dietary antioxidants to alleviate unwanted side effects from cancer therapy. Common side effects include anaemia, appetite loss and fatigue to name a few. Research surrounding alternative remedies to tackle cancer treatment effects have investigated polyunsaturated fatty acids, intact dairy protein, vitamins and minerals. The beneficial effect of marine protein hydrolysate intake during cancer therapy is warranted.

1.6 Anti-hypertensive activity of marine protein hydrolysates

A currently active area of marine protein hydrolysate research is that of angiotensin converting enzyme inhibition and anti-hypertensive activity and as such will be discussed (Harnedy and Fitzgerald, 2013). The aetiology of blood pressure dysregulation and subsequent hypertension is believed to play a vital role in other disease states, including cardiovascular, heart and kidney disease (Hermansen, 2013). The renal angiotensin system regulates blood pressure changes with several pharmaceutical targets identified for issues surrounding abnormal or chronically elevated blood pressure. Inhibition of the enzyme angiotensin-I-converting enzyme (ACE) has positive effects on reducing overall hypertension (Antonaccio, 1982). ACE functions by controlling and regulating the blood pressure and volumes of fluid within the body.

Angiotensinogen produced by the liver undergoes an enzymatic driven reaction that has subsequent effect on blood pressure. The body attempts to maintain homeostatic blood pressure, however increased activity of ACE increases sympathetic activity, increased reabsorption of Na⁺ and Cl⁻ with excretion of K⁺, arteriolar vasoconstriction, H₂O retention and subsequent elevation of blood pressure. This system undergoes acute changes and is counterbalanced with kidney negative feedback signals to promote excretion fluid. Antihypertensive drugs such as captopril and enalapril are potent ACE inhibitors currently available used in the treatment of hypertension and severe chronic heart failure (Packer *et al*, 1986). These work by inhibiting the conversion of angiotensin-I to angiotensin-II a vasoconstrictor and regulator of arterial blood pressure. The inhibition mechanism lies in the retardation or inhibition of the peptidyl-dipeptidase A, of which drives the angiotensin-I conversion. For over a decade now studies have identified foods that reduce hypertension in humans.

Particular interest has been generated for peptides derived from food proteins. Seaweed is a staple of Asian cuisine with intake associated with lower rates of hypertension (Wada *et al*, 2011). A study using 17 different kinds of proteases to generate ACE inhibitory hydrolysates from Wakame (*Undaria pinnatifida*) was assessed (Sato *et al*, 2002). Detecting hippuric acid liberated from Hip-His-Leu by ACE had a broad spectrum of inhibitory activity dependant on protease used in the protein hydrolysis process. Identified ACE inhibitors were then investigated in vivo using spontaneously hypertensive rats (SPR). Animals were grouped

and assigned a product which were comparative to control rats received dH₂O only. Only rats of which displayed >200 systolic blood pressure were used. Wakame protein hydrolysates were administered (10 - 100 mg/kg bodyweight) and subsequent SBP was measured before and up to 6 hours after oral administration of hydrolysates. Captopril (5 mg/kg bodyweight) was used as a positive control. Activity was again determined by proteolytic method deployed, however all hydrolysates had a somewhat positive effect on acute blood pressure lowering. SPH rats displayed markedly elevated systolic blood pressure after a few weeks after birth. Dietary addition of 0.1 and 1% wakame hydrolysate was able to significantly reduce systolic blood pressure versus standard rodent diet, respectively (Xie *et al*, 2014). Furthermore a study involving oyster protein hydrolysate displayed a reduction in SBP in SHR with separated di- and tripeptides inhibiting ACE activity with as little as 16.7, 29 and 51.2 μ M of peptide *in vitro*, respectively.

Further *in vitro* studies investigating the ACE inhibitory activity of cuttlefish wastewater hydrolysates (Amado *et al*, 2014), skate (*Okamejei kenojei*) skin gelatin (Ngo *et al*, 2014), thornback ray skin's gelatin (Lassoued *et al*, 2015) and smooth hound (*Mustelus mustelus*) waste biomass hydrolysates (Sayari *et al*, 2016), have shown a sweeping approach of potential dietary additions of unique and novel protein hydrolysates and biopeptides for the therapeutic management or treatment of hypertension.

1.7 Immunoregulatory effects of marine protein hydrolysates

It is of no surprise that researchers have a keen interest on immunological regulation and development via the use of dietary applications. The increasing aware of dietary influences and health are more pronounced now than ever. The therapeutic effect of specific foods has gained interested from food manufacturers and consumers, however, more importantly clinicians and researchers (Milner, 1999). The valorisation of macronutrients and their derivatives released during food metabolism is accelerated by the use of chemicals of which can digest, modify and potentially upgrade a food source for benefits above purely caloric value. Important functionalities include antimicrobial, antihypertensive, antioxidative, cytomodulatory and immunomodulatory effects (Lopez *et al*, 2016). Optimal immune functioning is vital for clearance or prevention of infection. This first line of defence against

pathogens can be altered according to health status. Growth, development and infection control are vital aspects of immune function, especially within the first several years of life (Simon *et al*, 2015). Investigation of immune system adaptation or development is investigated using an array of *in vitro* and *in vivo* approaches to track the regulation of immunomodulatory metabolites and the corresponding effect this may have on health. Very recently the area of nutritional intervention of dietary proteins to promote improved health and immune related health has improved. Reducing the risk of chronic disease or boosting the bodies innate and adaptive immune protection using dietary protein, peptides and amino acids has garnered scientific attention (Daly *et al*, 1990). Biologically active peptides derived from marine protein are now targets of immunomodulatory research.

Using seaweed protein hydrolysates from *Porphyra columbina*, researchers displayed an inhibition of pro-inflammatory cytokines, namely Interleukin-10 (IL-10), especially under lipopolysaccharide stimulation (Cian *et al*, 2012). On further investigation the hydrolysate did not increase production of lactate dehydrogenase with direct promotion of apoptotic pathways increased such as JNK, p38 and NF- κ B with immunomodulatory effects on macrophages and lymphocytes. Furthermore, using a systemic/chronic inflammation study using hTNF α mice (C57BL/6 hTNF α), (Bjørndal *et al*, 2013), this strain expresses chronic arthritis of the front and hind paws and a common model of study of chronically elevated inflammation. Animals were grouped and assigned a specific dietary regime for 2 weeks. The base diet consisted of high-fat (23%, w/w) with alterations to protein source of 20% casein (control) or 15% fish protein hydrolysate (FPH) and 5% casein (FPH group). On study completion liver, fatty acid composition, gene expression, blood glucose, adipose tissue were analysed. Results concluded that mice fed the FPH diet (15% FPH / 5% casein) displayed lower hepatic activity of fatty acid synthesis, furthermore INF- γ levels were lower, however levels of IL-1 β , IL-2, IL-5 and GM-CSF were unaffected by the diets. Inflammation was assessed using an antiinflammatory index with overall inflammation lower in FPH group versus control group after 2 weeks of intervention. Identification of peptides with immunomodulatory activity from biomass has shown capability of downregulating allergic responses *in vitro*. Understanding if marine protein hydrolysate administration has a causative negative effect on immune system was investigated using BALB/c mice, a strain commonly used for immunology and cancer and drug development studies (Duarte *et al*, 2006). Using a

fermentation process to liberate peptides from commercial fish protein was administered to animals at various concentrations (20-30 mg/ml) for several consecutive days. The study concluded that fish protein hydrolysate enhanced gut specific immunity without a pro-inflammatory outcome of which demonstrated capacity to enhance host defence systems.

Peptides identified from *Spirulina maxima* were shown to have suppressive effects of phospholipase C γ activation including reactive oxygen species inhibition. Furthermore, both identified biopeptides displayed the suppressive effects of IL-4 via nuclear factor- κ B translocation depression (Vo *et al*, 2014). The attempt to identify potentially potent bioactive marine peptides may be promising candidate for antiallergic therapeutics. Innate immunity and prospective disease resistance has its challenges of tracking accurately in humans. Using a controlled environment, groups of highly commercial fish species, Red Sea bream (*Pagrus major*) were fed various diets containing marine protein hydrolysates with growth performance, innate immunity and disease resistance analysed (Khosravi *et al*, 2015). Protein sourced from Krill, Shrimp and Tilapia were first hydrolysed. A broad spectrum of peptides ranging from 500 – 5000 da were produced. In total five isonitrogenous diets were formulated and fed to different groups of juvenile Red Sea Bream. The control diet contained high fish meal (HFM), whereas all other diets contained low fish meal (LFM) supplemented with soy protein (group B) or soy protein and various marine protein hydrolysates (group C (shrimp / Sh), D (Tilapia / TH) and E(Krill / KH)). Fish were hand-fed twice daily (09:00 and 17:00 h) for 13 weeks. Uneaten feed was subsequently collected and weighed. Growth performance was significantly lower in LFM group, whereas groups containing SH, TH and KH had marked growth rates, similar to that of the HFM diet group. Antioxidant capacity were significantly affected by dietary protein supplementation. Enhanced SOD activity was displayed across all hydrolysate groups comparative to LFM group. Several innate immune response parameters were significantly elevated versus LFM group such as lysozyme and antiprotease activities however there was no variance between the hydrolysate groups and HFM group. The study highlighted that a low fish meal diet supplemented with plant protein, specifically in this study, soy protein, had an adverse effect on growth, non-specific immune response, disease resistance and antioxidative capacity versus groups that were the same diet but addition marine protein hydrolysates added. The enhanced growth performance in diets supplemented with fish hydrolysates versus that of low fish meal based diet may have been

explained by the inclusion of bioactive peptides which were essential for the promotion of biological performance of the fish. These studies have demonstrated that protein, specifically hydrolysates and subsequent biopeptides have a role regulating or promoting immune system functioning and as such have a higher effect beyond merely nutrition.

1.8 Antimicrobial peptides derived from marine protein hydrolysates

Since the discovery and development of treatments specific to bacterial eradication within human systems the thought of bacteria endangering life was rumoured to be dramatically reduced. However, today, encroaching 90 years since the discovery of the first antibiotic, bacteria still represents a global threat (Ligon, 2004). The misuse of broad-spectrum antibiotics threatens to return modern healthcare to the pre-antibiotic era due to the rapid rise of multidrug resistant (MDR) bacteria. Therefore, the search for antimicrobial inhibiting molecules or antimicrobial peptides (AMPs) is now a priority. A growing class of AMPs are naturally occurring or synthetic peptides (Bahar & Ren, 2013)/(da Costa *et al*, 2015) or more recently generated via hydrolysis of protein. These AMPs are considered a new exploitable area of alternative hope to conventional antibiotics (Cheung *et al*, 2015). While naturally occurring AMPs represent host defence systems put in place over the course of an organisms evolution, it hasn't faltered researchers from delving into the mechanics of how these sequence of amino acids target and ultimately destroy microbes.

AMPs have diverse interaction and therapeutic use, dysregulation of endogenous peptides is notable in the lung disease, cystic fibrosis where the water and salt biofilm coated within the tracheobronchial epithelium fails to optimally function resulting in reduced activity of its host AMPs. Numerous AMPs have now been discovered with broad-spectrum antimicrobial activities that may be developed for therapeutic potential. Sources of current natural AMP interest are that of amphibian secretions (Conlon *et al*, 2009), however, high quality sources of marine protein after hydrolysis can yield an abundant quantity of small molecular weight peptides that are of current interest. Collagencin, an antibacterial peptide isolated from fish collagen was evaluated for its growth inhibitor activity of various different strains of bacteria (Ennaas *et al*, 2016). Collagencin was incubated with an array of G- and G+ bacteria at 235 μ M and resulted in inhibitory activity in several strains of bacteria including *S. pyogenes* ATCC19615, *E. coli* MC4100, *L. innocua* HPB29, *L. lactis* ATCC11454, and *C. divergens* M35 however failed to have any effect on *E. coli* O157:H7, *A. hydrophyla* ATCC7966, *P.*

aeruginosa ATCC27853, respectively. Collagenous materials, particular marine collagen are thought to be cost effective approach to treating bacterial spread due to containing an array of bioactive peptides locked within its primary structure. With this noted, fresh approaches of generating AMPs are using knowledge of peptide structure and mechanism of action and applying this to generate novel AMPs as potential therapeutic agents. An understanding of the hydrolysate optimisation techniques involved in generating large quantity of AMPs from novel sources of marine protein is first required.

1.9 Nutritional approaches of marine protein hydrolysates

Improving nutrition status is by far one of the most important aspects of improving overall health. The concept of food fortification was first conceived to improve dietary nutritional values, in turn this could be used to target areas of poor nutritional status and increase macronutrient, micronutrient, minerals or vitamin intake (Quinlivan & Gregory, 2003)/ (Kuong *et al*, 2016). The overall goal remains consistent, improvement of perceived health for this generation and the generations to come. Supplementation closely followed fortification, with the intake of higher quantities of a particular nutrient, particularly seen in exercising individuals who increase protein intake to aid in muscle development (Pasiakos *et al*, 2014). The global increase of nutritional knowledge and its impact on human health has led to significant discoveries surrounding nutrition, bioactive mining of health promoting compounds and mass production on an unprecedented scale to meet demand. The term functional food was coined for a food sources ability to improve general health or to ward of the risk of disease and illness and as such, has become one of the most expanding areas of consumer driven demand (Hesler, 2002).

This is to say that functional foods, or bioactive components, can also be used to fortify foods for additive nutritional value. Marine protein hydrolysates are again in the spotlight for their reported ability to improve various aspects of health including increasing bone mineral density, improving uptake calcium (Lin *et al*, 2015) and non-haem iron absorption (Li *et al*, 2017) and ability to improve performance and growth (Thuy & Ha, 2016). Using ovariectomised rats, orally administered small molecular weight peptides generated from

shark gelatine hydrolysate (SGH) resulted in increased femur bone mineral density versus control group. Furthermore, the bone content of type I collagen and glycosaminoglycan, analysed via western blot, were increased in the SGH group versus control, respectively (Nomura *et al*, 2005). Explaining the bone promoting benefits of marine protein hydrolysates has recently evolved. Hydrolysates generated with smaller molecular weight peptides display improved mineral and trace element chelating properties (Cai *et al*, 2017).

A study investigating hydrolysates derived from microalgae (*Schizochytrium sp*) tested 22 fractions separated via RP-HPLC. Of the 22 chromatograph peaks collected, 17 displayed calcium chelating properties. In particular a specific peptide Tyr-Leu (YL) displayed calcium binding capacity of approximately 130 ug of calcium per mg of peptide and displayed excellent thermal stability and solubility. When peptide YL-Ca was incubated in CaCo-2 cell, it improved calcium uptake and reduced calcium precipitation due to other reductive nutritional factors. Results displayed marine protein hydrolysates can improve calcium uptake and may be used in the novel treatment of illnesses such as osteoporosis.

1.10 Discussion & Conclusion

Marine protein and its subsequent hydrolysed or identified bioactive peptides used in this review have displayed insight into their therapeutic potential. Identifying problematic areas of modern day health care and its current approach and potential novel incorporation of what is essentially food derived molecules that present potent bioactivity across *in vitro*, *in vivo* and human study's using various disease states. Marine protein can contain an almost unlimited source of stored bioactive peptides which can be unlocked using various hydrolysis approaches. The approach to generating bioactive hydrolysates may use the classical extremes of high or low pH solutions, however it is clear that the current general consensus is the use of proteolytic enzymes. Furthermore, enzymes used sequentially can yield a great number of bioactive peptides within a specific molecular weight target. Delving deeper into the crude hydrolysates using identification methods of individual biopeptides, this review highlighted the current methods deployed with the target molecule of interest playing a vital role in the analytical approaches deployed. Methods such as size exclusion chromatography or capillary electrophoresis have their place but the most controllable, high product yielding

and further up-scalable approach of separation of crude hydrolysate fractions or single peptides is that of RP-HPLC.

The areas covered within this review are a brief up-to-date insight to the potentially rewarding therapeutic areas of marine research, in particular the emerging area of marine protein hydrolysates. A large number of generated hydrolysates and their identified peptide components have displayed potent anti-hypertensive, anti-obesity, antidiabetic, antimicrobial and so forth. Furthermore, expanding areas of marine protein hydrolysates research are treatment of hyperlipidaemia, mineral and trace element chelating properties, improvement of growth performance and improvement of sports performance in athletes. This review aims to serve as a guide and gather information for the bioactive screening approach required to successfully generate marine protein hydrolysates with potential therapeutic activity.

It is noted that several factors must be met in order to streamline the screening process. The quality, however not limited by quantity of initial intact protein source, followed by the protease/s used in the digestion process. Using several proteases that complement each other, particularly protease activity at certain pH and temperature ranges can improve the degree of intact protein hydrolysis and finally the separation, fractioning and identification methods deployed. Once each of these outcomes are successfully satisfied, screening bioactivity using an array of *in vitro*, *in vivo* and human study can begin. The study highlighted certain therapeutic applications of marine protein hydrolysates favoured certain proteases used in the hydrolysis procedure, molecular size and hydrophobicity profile.

It is understandable that smaller di- and tripeptides have increase absorption rates, however using a broad-spectrum of small molecular weight peptides (2 to 20 amino acids) may have advantages and via potentially targeting multiple therapeutic areas. Furthermore, the ability of bioactives to ultimately improve health can work in two main directions, the direct bioactivity reported within in this review via metabolism via hormonal regulation or secretion, and secondly, simply used as a nutritional source of high quality nitrogenous macronutrients.

Chapter 2

Materials & Methods

2.1 Hydrolysates preparation

Palmaria palmata (Dulse)

Sample preparation by collaborators at Limerick University

A sample of air-dried milled (5 mm) *P. palmata* sample was purchased from Irish Seaweeds Ltd., Belfast, Co. Antrim, Northern Ireland. The macroalgae was further milled with a Cyclotec™ Mill (1 mm screen, FOSS Tecator AB, Hoganas, Sweden) and subsequently stored at room temperature.

Extraction and quantification of aqueous and alkaline soluble proteins

Crude aqueous and alkaline soluble protein extracts were prepared using the method described by Harnedy *et al.* (2013a) with some modifications. In brief, the milled *P. palmata* powder was suspended at a mass:volume ratio of 1:20 ((w/v), 1 kg:20.0 L) and gently stirred at room temperature for 3 h. The supernatant containing the aqueous soluble protein was obtained following centrifugation at 4,190 x g (Sorvall RC6 Plus, Fisher Scientific, Dublin, Ireland) for 15 min at room temperature. The pellet was resuspended in 0.12 M NaOH (1:15 (w/v)) and gently stirred for 1 h at room temperature and the supernatant containing the alkaline soluble protein was obtained following centrifugation as described above. The pellet from the above was subjected to a second alkaline extraction using the same conditions and both supernatants were combined. The aqueous and alkaline soluble protein components were semi-purified and concentrated by a double isoelectric precipitation step at pH 2.5 (aqueous) and 8.0 (alkaline), respectively using 1.0 M HCl. The precipitated protein pellets obtained following the second isoelectric precipitation were resuspended in distilled water

(dH₂O) to a protein concentration of ~ 2.4% (w/v) and combined. The concentration of protein in the extracts was determined by the modified Lowry protein quantification method as described previously (Harnedy & FitzGerald, 2013b). All samples were analysed in triplicate.

Enzymatic hydrolysis of macroalgal proteins

Macroalgal protein was hydrolysed by the method described by Harnedy *et al.* (2013a). A 2% (w/v) protein solution was preheated to 50°C and adjusted to pH 7.0 and hydrolysed with Alcalase 2.4L, Alcalase 2.4L and Flavourzyme 500L, Bromelain and Promod 144MG at an enzyme:substrate (E:S) ratio of 1:100 (w/w or v/w) for 4 h at 50 °C. The hydrolysis reaction was maintained at pH 7.0 using a pH-stat (842 Titrand, Metrohm, Switzerland) and terminated by heating at 90°C for 20 min. A control protein sample, containing no proteolytic enzyme, was treated in the same manner. All samples (control and hydrolysates) were subsequently freeze-dried (FreeZone 18L, Labconco, MO, USA) and stored at -20°C.

Simulated gastrointestinal digestion (SGID)

The unhydrolysed protein control and hydrolysates were subjected to simulated gastrointestinal digestion (SGID) as described by Walsh *et al.* (2004) with modifications. In brief, unhydrolysed protein controls and hydrolysates were diluted to 2.0% (w/v) protein in water and incubated at 37°C and pH 2 for 90 min with pepsin at an E:S of 1:40 (w/w). The samples were altered to pH 7 and subjected to heat inactivation at 90°C for 20 min. The samples were incubated for a further 150 min at 37°C with Corolase PP (E:S of 1% (w/w)). This simulated gastrointestinal digestion sample was inactivated as described above. All samples were subsequently freeze-dried (FreeZone 18L, Labconco, MO, USA) and stored at -20°C.

Atlantic salmon (*Salmo salar*) gelatin and trimmings

Sample preparation for both salmon gelatin and trimmings

Samples of Atlantic salmon (*Salmo salar*) trimmings and skin were kindly provided by The Good Fish Processing Company Ltd., Carrigaline, Co. Cork. The skins were stored at -20°C until required. The trimmings were minced using a Meat Mincer equipped with a 1.5 cm diameter perforated disk (Breville, Oldham, UK) and were also stored at -20°C.

Extraction of gelatine from salmon skins

The thawed skins were cut into pieces (about 5×5 cm) and washed by stirring in distilled water (1:5 (w/v)) for 15 min. Salmon skins were separated by filtration using a double layer of cheese cloth between each step of the extraction procedure. The fish skins were then soaked in 0.2 M NaOH (1:5 (w/v)) and stirred at room temperature for 15 min to remove non-collagenous proteins. This procedure was repeated three times. NaOH was removed by soaking the skins for 15 min in distilled water at a mass:volume ratio of 1:5 (w/v) until the pH was neutral (repeated 3 times). The skins were then suspended in distilled water (1:6 (w/v)), the pH was altered to pH 3 with 1M HCl, and stirred for 1 h at room temperature. Again the removal of HCl was performed by soaking the skins for 15 min in distilled water (1:5 (w/v)) until the pH was neutral (repeated 3 times). The gelatine in the swollen skins was then extracted in distilled water (1:5 (w/v)) at 50°C for 16 h. The supernatant was freeze-dried and stored at -20°C until required.

Protein quantification

The protein content of salmon gelatine and purity of the hydrolysates generated were quantified using a macro-Kjeldahl procedure (Connolly *et al.* 2013). The nitrogen to protein

conversion factor used was 5.55 (FAO/INFOODS, 2012). All samples were analysed in triplicate.

Enzymatic hydrolysis of salmon skin gelatine

Salmon skin gelatine was hydrolysed by the method described by Harnedy & FitzGerald (2013a) with some modifications. A 7% (w/v) gelatine solution was preheated to 50°C and adjusted to pH 7.0 and hydrolysed with Alcalase 2.4L, Alcalase 2.4L and Flavourzyme 500L, Bromelain and Promod 144MG at an enzyme:substrate (E:S) ratio of 0.73% (w/w or v/w) at 50°C. The hydrolysis reaction was maintained at pH 7.0 using a pH-stat (842 Titrando, Metrohm, Switzerland). Samples were taken at 1, 2, 3 and 4 h and terminated by heating at 90°C for 20 min. A control protein sample, containing no proteolytic enzyme, was treated in the same manner. All samples (control and hydrolysates) were subsequently freeze-dried (FreeZone 18L, Labconco, MO, USA) and stored at -20°C.

Generation of salmon trimming meat protein hydrolysates by direct enzymatic hydrolysis

The minced salmon trimmings were suspended at 1:1.75 (w/v) in distilled water and homogenised at 24,000 rpm/min for four x 15 sec durations (Ultra-Turrax® T25 Basic, IKA®, Staufen, Germany). Additional water was added following homogenisation to obtain a 6.83% (w/v) salmon meat protein suspension. The salmon protein suspension was preheated to 50°C and adjusted to pH 7.0 and hydrolysed by three separate means with Alcalase 2.4L, Alcalase 2.4L and Flavourzyme 500L or Promod 144MG at an enzyme:substrate (E:S) ratio of 1:135

(w/w or v/w) at 50°C. The hydrolysis reaction was maintained at pH 7.0 using a pH-stat (842 Titrando, Metrohm, Switzerland). The hydrolysates were terminated after 4 h by heating at 90°C for 20 min. A control protein sample, containing no proteolytic enzyme, was treated in the same manner. Peptides were then separated by double filtration (Whatman grade 1: 11 µm). All samples (control and hydrolysates) were subsequently freeze-dried (FreeZone 18L, Labconco, MO, USA) and stored at -20°C.

Protein quantification

The protein content of salmon trimming meat and protein content and purity of the hydrolysates generated were quantified using a macro-Kjeldahl procedure (Connolly *et al.* 2013). The nitrogen to protein conversion factor used was 6.25 (Kristinsson and Rasco, 2000). All samples were analysed in triplicate.

SGID: Homogenised salmon trimming meat and hydrolysates were subjected to SGID as above (section 2.1)

Blue whiting

Sample preparation

Samples of minced blue whiting (*Micromesistius poutassou*) meat were kindly provided by *Killybegs Fishermen's Organisation*, Killybegs, Co Donegal, Ireland through Bord Iascaigh Mhara (BIM, Ireland) and stored at -20°C.

Generation of blue whiting hydrolysate by direct enzymatic hydrolysis

The minced blue whiting meat was suspended at 1:1.25 (w/v) in distilled water and homogenised at 24,000 rpm/min for four x 15 sec durations (Ultra-Turrax® T25 Basic, IKA®, Staufen, Germany). Additional water was added following homogenisation to obtain a 6.00% (w/v) blue whiting protein suspension. The suspension was preheated to 50°C and adjusted to pH 7.0 and hydrolysed with Alcalase 2.4L and Flavourzyme 500L at an enzyme:substrate (E:S) ratio of 1:135 (v/w) at 50°C. The hydrolysis reaction was maintained at pH 7.0 using a pH-stat (842 Titrand, Metrohm, Switzerland). The enzymes were inactivated after 4 h by heating at 90°C for 20 min. Peptides were then separated by double filtration (Whatman grade 1: 11 µm), freeze-dried (FreeZone 18L, Labconco, MO, USA) and stored at -20°C.

SGID: Homogenised blue whiting meat and the hydrolysate were subjected to SGID as explained above (Section 2.1).

Protein quantification

The protein content of blue whiting meat and protein content and purity of the hydrolysates generated were quantified using a macro-Kjeldahl procedure (Connolly *et al.* 2013). The nitrogen to protein conversion factor used was 6.25 (Kristinsson and Rasco, 2000). All samples were analysed in triplicate.

Boarfish (Lab scale samples)

Sample preparation

Samples of minced boarfish (*Capros aper*) meat were kindly provided by *Killybegs Fishermen's Organisation*, Killybegs, Co Donegal, Ireland through Bord Iascaigh Mhara (BIM, Ireland) and stored at -20°C.

Generation of a boarfish hydrolysate by direct enzymatic hydrolysis

The minced boarfish meat was suspended at 1:1 in distilled water and homogenised at 24,000 rpm/min for four x 15 sec durations (Ultra-Turrax® T25 Basic, IKA®, Staufen, Germany). Additional water was added following homogenisation to obtain a 6.83% (w/v) boarfish protein suspension. The suspension was preheated to 50°C and adjusted to pH 7.0 and hydrolysed with Alcalase 2.4L and Flavourzyme 500L at an enzyme:substrate (E:S) ratio 1:150 (v/w) at 50°C. The hydrolysis reaction was maintained at pH 7.0 using a pH-stat (842 Titrando, Metrohm, Switzerland). The hydrolysates were terminated after 4 h by heating at 90°C for 20 min. Peptides were then separated by double filtration (Whatman grade 1: 11 µm). All samples (control and hydrolysates) were subsequently freeze-dried (FreeZone 18L, Labconco, MO, USA) and stored at -20°C.

SGID: Homogenised boarfish meat and the hydrolysate were subjected to SGID as described above (Section 2.1).

Kjeldahl nitrogen quantification method

The protein content of the boarfish meat and protein content and purity of the hydrolysates generated were quantified using a macro-Kjeldahl procedure (Connolly *et al.* 2013). The nitrogen to protein conversion factor used was 6.25 (Kristinsson and Rasco, 2000). All samples were analysed in triplicate.

Boarfish (Semi-pilot scale)

Sample preparation

Samples of minced deboned boarfish (*Capros aper*) meat were kindly provided by *Bio-Marine Ingredients Ireland Ltd*, Killybegs, Co. Donegal, Ireland.

The boarfish protein hydrolysate was generated at semi-pilot scale as per the method used to generate the hydrolysate at laboratory scale with modifications. In brief, a boarfish suspension (1900 kg) with a protein content of 6.0 % (w/v) incubated at 50°C was adjusted to pH 7.0 and hydrolysed with Alcalase 2.4L and Flavourzyme 500L at an enzyme:substrate (E:S) ratio of 1:150 (v/w). The hydrolysis reaction was maintained at pH 7.0. After 4 h hydrolysis, the enzyme was inactivated at 90°C. The protein/peptide component was separated from the sediment and oil by a 2 step decanter/centrifuge process. The oil and sediment were first separated from the supernatant using a 3-phase decanter (*GEA Westfalia Separator Group GmbH*). The supernatant was then further clarified using a continuous centrifuge (*GEA Westfalia Separator Group GmbH*) and cooled to <4°C overnight. The hydrolysate was pasteurised at 85°C for 85 sec and fed into a 3 stage falling film evaporator and evaporated to ~40% total solids. The evaporated hydrolysate was spray dried (80 kg/h water evaporation) using an inlet and outlet temperature of 180 °C and 90°C, respectively and stored in an airtight container at 4°C until required.

Table 1 – List of hydrolysates generated at UL and sent to UU for bioactivity analysis

Raw material	Sample No	substrate	Enzyme	Time (h)	SGID	
Salmon skin gelatin	ULPH032	Salmon skin gelatin - Control 4h: and subjected to SGID	Pepsin/Corolase PP		SGID of ULPH001	
	ULPH024	Salmon skin gelatin - Alcalase 4h: and subjected to SGID	Pepsin/Corolase PP		SGID of ULPH005	
	ULPH026	Salmon skin gelatin - Alc+Flav 4h: and subjected to SGID	Pepsin/Corolase PP		SGID of ULPH005	
	ULPH028	Salmon skin gelatin - Brom 4h: and subjected to SGID	Pepsin/Corolase PP		SGID of ULPH017	
	ULPH030	Salmon skin gelatin - Prom 4h: and subjected to SGID	Pepsin/Corolase PP		SGID of ULPH013	
	Palmaria palmata	ULPH018	Palmaria palmata aq+alk protein	Control	4	
ULPH019		Palmaria palmata aq+alk protein	Alcalase	4		
ULPH020		Palmaria palmata aq+alk protein	Alcalase + Flav	4		
ULPH021		Palmaria palmata aq+alk protein	Bromelain	4		
ULPH022		Palmaria palmata aq+alk protein	Promod 144 MG	4		
ULPH042		Palmaria palmata - Control 4h: and subjected to SGID	Pepsin/Corolase PP		SGID of ULPH018	
ULPH034		Palmaria palmata - Alcalase 4h: and subjected to SGID	Pepsin/Corolase PP		SGID of ULPH019	
ULPH036		Palmaria palmata - Alc+Flav 4h: and subjected to SGID	Pepsin/Corolase PP		SGID of ULPH020	
ULPH038		Palmaria palmata - Brom 4h: and subjected to SGID	Pepsin/Corolase PP		SGID of ULPH021	
ULPH040		Palmaria palmata - Prom 4h: and subjected to SGID	Pepsin/Corolase PP		SGID of ULPH022	
Salmon trimmings hydrolysates		ULPH051	salmon trimmings - direct hydrolysis with Alcalase	Alcalase	4	
		ULPH052	salmon trimmings - direct hydrolysis Alcalase + Flav	Alcalase + Flav	4	
		ULPH053	salmon trimmings - direct hydrolysis with Promod	Promod 144 MG	4	
		ULPH057	salmon trimmings- Alcalase 4h: and subjected to SGID	Pepsin/Corolase PP		SGID of ULPH051
	ULPH059	salmon trimmings - Alc+Flav 4h:and subjected to SGID	Pepsin/Corolase PP		SGID of ULPH052	
	ULPH061	salmon trimmings - Prom 4h: GI phase:and subjected to SGID	Pepsin/Corolase PP		SGID of ULPH053	
	salmon trimmings	ULPH055	salmon trimmings - direct hydrolysis with pepsin and Corolase PP (GI phase) seperated by centrifugation	Corolase PP		Direct SGID of salmon trimmings
ULPH064		salmon trimmings- Alcalase 4h: GI phase (repeat of ULPH057)	Pepsin + Corolase PP	1.5/2.5		
Boarfish	ULPH062	Boarfish - direct hydrolysis with Alcalase + Flav	Alcalase + Flav	4		
	ULPH068	Boarfish - direct hydrolysis with Alcalase + Flav and subjected to SGID	Pepsin /Corolase PP		SGID of ULPH062	
	ULPH066	Boarfish - direct hydrolysis with SGID enzymes Pepsin/Corolase PP	Pepsin /Corolase PP			
	ULPH074	Boarfish - direct hydrolysis with Alcalase + Flav at semi-pilot scale	Alcalase + Flav	4		
Blue whiting	ULPH063	Blue whiting - direct hydrolysis with Alcalase + Flav	Alcalase + Flav	4		
	ULPH070	Blue whiting - direct hydrolysis with Alcalase + Flav and subjected to SGID	Pepsin /Corolase PP		SGID of ULPH063	
	ULPH072	Blue whiting - direct hydrolysis with SGID enzymes Pepsin/Corolase PP	Pepsin /Corolase PP			

2.2 Materials

Cell culture materials were obtained from Gibco life technologies (Thermo Fisher Scientific, Paisley, UK) including RPMI-1640 cell culture media (BRIN-BD11), DMEM-4500 mg/L glucose cell culture media (STC-1) DMEM-1000 mg/L cell culture media (GLUTag) and penicillin streptomycin (10000 U/ml, 10000 µg/L). Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), D-glucose, hydrochloric acid (HCl), magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), sodium chloride (NaCl), Dimethylsulphoxide (DMSO), biological buffer sodium salt (HEPES), potassium chloride (KCl) and sodium hydroxide (NaOH) were obtained from Sigma-Aldrich Chemical Company Ltd. (Dorset UK). Controls used for acute secretory activity, glucagon-like peptide-1 (GLP-1 7-36_{amide}) was obtained from EZbiolabs (Indiana, USA), L-Glutamine ($\text{C}_5\text{H}_{10}\text{N}_2\text{O}_3$) and palmitic acid ($\text{CH}_3(\text{CH}_2)_{14}\text{COOH}$) were obtained from Sigma-Aldrich Chemical Company Ltd. (Dorset UK).

2.1.2 Cell culture

The maximum hydrolysate concentration of 2.5 mg/ml was used in acute secretory activity in all cell lines. This was further serially diluted to assess the dose-dependent activity to a minimum dose of 0.039 mg/ml of chosen protein hydrolysate.

2.1.3 BRIN-BD11 rat pancreatic β -cell culture

BRIN-BD11 cells represent a robust rat insulin-secreting cell line which responds to macronutrients such as glucose and glucose derivatives, peptide hormones and pharmacological agents. These cells were established after electrofusion of RINm5f cells with New England Deaconess Hospital rat pancreatic islet cells. Morphological studies established that BRIN-BD11 cells grow as monolayers maintaining stability in tissue culture settings for

45- 50 passages (McClenaghan *et al.* 1996). An important immortalised and stable glucose-responsive cell line. BRIN-BD11 cells were cultured until passage 25 where they were cryopressed in 1 ml vials (Sterilin Ltd, Houslow, UK) containing 1×10^6 cells/ml in freezing media (10% RPMI-1640 media, 10% DMSO & 80% FBS). Cells were firstly stored at 20°C for 4 h then transferred to -80°C for 12 h before finally being transferred to liquid nitrogen storage (-196°C) for future use.

2.1.4 GLUTag mouse enteroendocrine cell culture

GLUTag cells generated by the Daniel Drucker laboratory in Toronto and gifted to Ulster University via Prof. Gribble at Cambridge University are immortalized. GLUTag cells are a relatively well differentiated murine enteroendocrine cell line that express the proglucagon gene and secrete glucagon-like peptides in a regulated manner. The GLUTag cell line was isolated from a glucagon-producing enteroendocrine cell tumour that arose in glucagon gene-SV40 T antigen transgenic mice. The production of the large bowel carcinoma expressing the glucagon gene was then serially passaged in nude mice which gave rise to glucagonomas that exhibit a stable phenotype of glucagon gene expression. GLUTag cells are extensively utilized for the analysis of glucagon-like peptide 1 & 2 secretagogues and for investigation of enteroendocrine glucagon gene transcription as well as to a lesser extent neurotensin (NT), Glucagon, cholecystokinin (CCK) and somatostatin (SST) (Brubaker *et al.* 1998). This cell line was chosen due to its responsiveness to peptides, amino acids, pharmacological agents and fatty acids that stimulate secretory activity. GLUTag cells were received from Cambridge University at passage 9, cells were cultured by Ulster University until passage 12 where they were cryopressed in 1 ml vials (Sterilin Ltd, Houslow, UK) containing 1×10^6 cells/ml in freezing

media (90% DMEM media, 10% DMSO).. Cells were firstly stored at -20°C for 4 h then transferred to -80°C for 12 h before finally being transferred to liquid nitrogen storage (-196°C) for future use.

Experimental GLUTag cells were cultured in Dulbecco's modified eagle medium (DMEM) devoid of glutamine and supplemented with glucose (5 g), fetal bovine serum (10%) and penicillin streptomycin (100 µmol/ml & 0.1 mg/ml streptomycin) in a sterile breathable however filtered 75 cm² tissue culture flask until a cell confluence of 70% was reached. Cells were then gently washed in 10 ml of HBSS prior to incubating with 3 ml of pre-warmed 1% trypsin/EDTA at 37°C for 5 – 8 min. Detached cells were observed using a phase contrast microscope (Zeiss, Germany). GLUTag cells grow in clusters, thus several aspirations of the trypsinized cells are required to gently break clusters to provide single cells for counting. The trypsin was neutralized using DMEM media and transferred to a sterile polypropylene tube (Sterlin Ltd, Hounslow, UK) vial for centrifugation at 900 rpm for 5 min. Cell supernatant was then removed and cells are re-suspended in a known volume of pre-warmed DMEM media. A single aliquot of 100 µl of re-suspended cells were mixed with 100 µl of Trypan blue and transferred for counting to a Neubauer haemocytometer (Scientific Supplies Co., Middlesex, UK). GLUTag cells were either transferred to a T75 tissue culture flask for future use or seeded onto a 24 well plate at a cell density of 1.5×10^5 for GLP-1 secretion studies.

2.1.5 STC-1 mouse enteroendocrine cell line

The STC-1 cell line was derived from a C57B1/6J mouse of which developed an invasive small intestinal neuroendocrine carcinoma. The overall cellular morphology of the STC-1 is that of

several different enteroendocrine cell types derived from the tumour originally excised from the animal. Several studies have vigorously screened the secretory activity of cultured STC-1 cells and their nutrient sensing capabilities (Kuhre *et al*, 2016; Gillespie & Green, 2016). The STC-1 cell line has the ability to secrete glucose-dependant insulinotropic polypeptide (GIP), glucagon-like-peptide 1 (GLP-1), glucagon-like-peptide 2 (GLP-2), Cholecystokinin (CCK), Oxyntomodulin and Peptide YY, making this cell line a favourable source for potentially assessing satiating compounds with anti-diabetic activity. STC-1 cells are characterised as relatively slow growing cells (45-56 h proliferation time) in standard growth media. STC-1 cells were cultured in supplemented DMEM media (DMEM containing 4.5 g/l D-glucose, without sodium pyruvate) (GlutaMAX, GIBCO, Paisley, UK) with 17.5 % foetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/l streptomycin and incubated in a 5% air humidified atmosphere at 37°C. Cells were passaged at 80–90 % confluence.

Experimental STC-1 cells were cultured in DMEM (supplementary additions mentioned above) in a sterile breathable however filtered T75 tissue culture flask until a cell confluence of 90% was reached. Cells were then gently washed in 10 ml of HBSS prior to incubating with 3 ml of pre-warmed 1% trypsin/EDTA at 37°C for 5 – 8 min. Detached cells were observed using a phase contrast microscope (Zeiss, Germany). As STC-1 reach confluence they grown in clusters, several aspirations of the trypsinized cells were required to gently break clusters to provide single cells for counting. The trypsin was neutralized using DMEM media and transferred to a sterile polypropylene tube (Sterlin Ltd, Hounslow, UK) vial for centrifugation at 900 rpm for 5 min. Cell supernatant was then removed and cells are re-suspended in a known volume of pre-warmed DMEM media. A single aliquot of 100 µl of re-suspended cells were mixed with 100 µl of Trypan blue and transferred for counting to a Neubauer

haemocytometer (Scientific Supplies Co., Middlesex, UK). STC-1 cells were either transferred to a T75 tissue culture flask for future use or seeded onto a 24 well plate at a cell density of 1.5×10^5 for secreting studies.

2.2 Cell secretion and analysis

2.2.1 Determination of insulin secretion from BRIN-BD11 cell cultures

The insulin radioimmunoassay (RIA) was used for the determination of insulin from both insulin secreting cell lines and from *ex-vivo* blood plasma samples (Flatt & Bailey, 1981). Initially BRIN-BD11 cells were seeded onto 24 well plates and incubated with compounds of interest for 1 hand subsequently collected for later analysis (described in 2.2.3) using RIA. In brief cells are seeded at a cell density of 1.5×10^5 cell/well in 1 ml of RPMI-1640 cell culture media and left for 21 h to adhere. On the day of experimentation media was removed and replaced with 1 ml of priming KRBB (11 mM NaCl, 4.7 mM KCl, 1.28 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.2 mM KH_2PO_4 , 1.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mM NaHCO_3 , 25 mM HEPES, 0.1% (w/v) bovine serum albumin, pH 7.4) and supplemented with 1.1 mM glucose at 37°C for 40 min. The entire cell prepping KRBB (1.1 mmol/L glucose) was then removed and further supplemented with KRBB of which contains at least 5.6 mmol/L glucose in all conditions (baseline) with compounds of interest used in a dose dependant manner minimum of n=8 replicates. After 20 min of co-incubation with compounds of interest 900 μl was removed from each well and stored until analysed by insulin radioimmunoassay at -20°C.

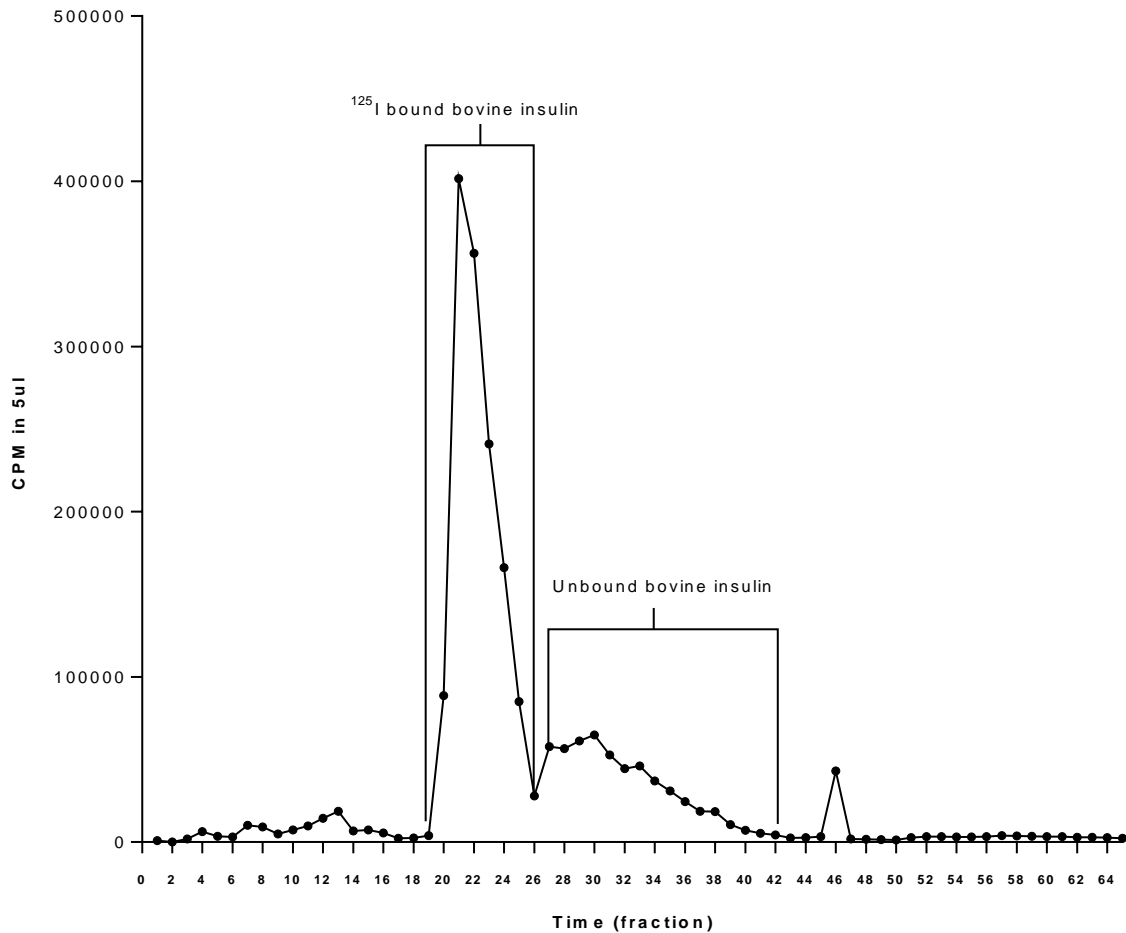
2.2.2 Preparation of iodinated bovine insulin for radioimmunoassay

Iodination of insulin for use in competitive enzyme binding assays has been in use for several decades due to its high sensitivity, low sample volume required and reproducibility. Insulin was iodinated according to the protocol by **Fracker and Speck (1978)**. Lyophilised bovine insulin (1 mg/ml) was dissolved in 10 mM HCl then diluted (1:8) in 500 mM sodium phosphate buffer to a concentration of 125 µg/ml. When needed and directly before use stock iodogen (1,3,4,6-Tetrachloro-3α,6α-diphenylglycouril) was first prepared as 2 mg/ml in dichloromethane (CH₂Cl₂). Once dissolved, 20 µl iodogen was then pipetted to the bottom of a clear 1.5 ml Eppendorf tube. With the lid left open the bottom of the tube was placed into a water bath very briefly (30 – 60 sec) then removed and rotated until a thin coat is remaining at the bottom of the tube. Iodogen tubes were stored in a desiccator for up to 1 month, however for optimal iodination it is advised to prepare fresh directly before use. Within the RIA controlled area (the operator wore 2 pairs of gloves, and lead screening) the reaction between ¹²⁵I and insulin was initiated by first placing 5 µl of ¹²⁵I (18.5 mbq / 0.5 mCi), followed by 20 µl insulin (125 µg/ml) into the iodogen coated Eppendorf tube which was placed on ice. Over the next 15 min the Eppendorf underwent gentle agitation using an index finger every few min. After the 15 minutes are complete the total 25 µl (¹²⁵I & insulin) are transferred to a fresh Eppendorf tube. The old tube was carefully washed with 500 µl of sodium phosphate buffer (50 mM) and mixed into the new tube then subsequently separated by RP-HPLC for separation of unbound and insulin bound ¹²⁵I.

RP-HPLC was deployed using a TSP Spectra RP-HPLC system (Thermo Finnigan, NJ, USA) equipped with a dual gradient TSP P200 solvent proportioning pump, Rheodyne 7125 injection valve with 500 µl loop and a Vydac C18 analytical reverse phase column (250 x 4.6

mm) (Hesperia, CA, USA). The Vydac column was equilibrated (1.0 ml/min flow rate) prior to injection using a 45 min gradient method consisting of deionized H₂O + trifluoroacetic acid (0.12% v/v (CF₃CO₂H) (Sigma, Dorset, UK) (Solvent A) and deionized H₂O (29.9%), acetonitrile (70%) + TFA (0.1% v/v) (Solvent B). The elution of ¹²⁵I Insulin was completed using the same approach, on injection the gradient of solvent B was raised from 0% to 40% over 10 min and from 40% to 80% over 40 min and finally from 80% to 100% for 10 min, during this run a Bio-Rad fraction collector (Model 2110) (Bio-Rad, Hertfordshire, UK) containing 72 x 10 mm polypropylene tubes was set at 1 min intervals collecting the entire 60 min run. Unbound ¹²⁵I was typically eluted at fraction 4, whereas the ¹²⁵I bound Insulin will be eluted within fractions 20 to 25 with unbound insulin eluted within fraction 27 – 32. An aliquot of each fraction (5 µl) was finally tested for counts per minute (CPM) from each fraction using a multigamma counter (LKB Wallac, Finland). A plot was drawn for CPM versus time (min), with each min representing the fraction collected during the RP-HPLC separation process. On completion of establishing the count per minute a graph was produced using GraphPad™ Prism 5 with the highest CPM counts being stored and diluted in a 1:1 ratio with sodium phosphate buffer (40 mM, pH 7.4) containing 1% (w/v) BSA and 1.2 mg/ml thimersol. Collected and diluted fractions were kept at 2-6°C prior to assessment of antibody binding by radioimmunoassay. Fractions with similar percentage binding were pooled for use with desired binding percentage based on antibody concentration, typically 1:25000 to 1:65000 with a binding percentage of 40% desirable.

Fig 1. ¹²⁵I bovine insulin vs unbound bovine insulin



2.2.3. Determination of insulin concentration by radioimmunoassay

Sample insulin content was measured by dextran-coated charcoal radioimmunoassay (Flatt and Bailey, 1981), using crystalline rat insulin standard, guinea-pig anti-porcine antiserum and ¹²⁵I bovine insulin standard. Initially stock RIA buffer consisted of 40 mM disodium hydrogen orthophosphate containing 0.3% (w/v) sodium chloride and 0.02% thimersol titrated using 40 mM sodium dihydrogen orthophosphate to pH 7.4. Working RIA buffer included the addition of 0.5% bovine serum albumin (w/v). Insulin standard curves were prepared in triplicate (200 µl) by serial dilution of rat insulin standard from 20 ng/ml stock concentration to 0.039 ng/ml in KRBB supplemented with 0.1% (w/v) BSA. Unknown insulin content from cell supernatant samples were added in duplicate (200 µl) while insulin standards receive 100 µl of guinea pig

anti-porcine (antibody binding concentration determined prior insulin antibody being diluted) followed by the addition of 100 μ l of 125 I bovine insulin (10,000 cpm/ 100 μ l). Samples were then incubated for 72 h at 4°C.

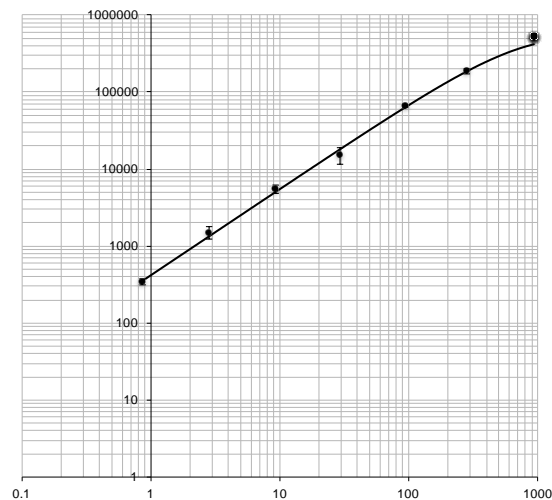
Detection of antibody bound insulin was determined by the addition of 1 ml of 5% dextran T-70 coated charcoal (dilution of 1:5 with stock RIA buffer) to each tube and incubation for 20 min at 4°C. Following incubation, the samples were centrifuged at 2500 rpm for 20 min. The supernatant was decanted and the radioactivity contained within the pellet (corresponding to unbound 125 I insulin) and the pellet was measured using a 1261 Multigamma counter (LKB, Wallac, Finland) linked to DOS-CPM software enabled computer. Insulin concentration in unknown samples were determined from the insulin standard curve (prepared using known rat insulin concentration standards), created using spline-fitting algorithm.

2.2.4. Determination of glucagon-like-peptide 1 (GLP-1) concentration by ELISA

GLP-1 is secreted by intestinal L-cells after oral ingestion of nutrients from food. It plays an important role in metabolic homeostasis with the bioactivity of GLP-1 being reduced in obese subjects or those with type 2 diabetes. Using mouse enteroendocrine cell culture (GLUTag culturing protocol in detail, 2.1.4) the experimental procedure to determine GLP-1 content in cell supernatant or biological samples was as follows. Initially GLUTag cells were seeded onto 24 well plates and incubated with compounds of interest for 2 hour and subsequently collected for later analysis. In brief cells were seeded at a cell density of 1.5×10^5 cell/well in 1 ml of DMEM 1000 mg/mL glucose cell culture media and left for 48 h to adhere. On the day of experimentation, media was removed and replaced with 1 ml of

priming KRBB (11 mM NaCl, 4.7 mM KCl, 1.28 mM CaCl₂·2H₂O, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 10 mM NaHCO₃, 25 mM HEPES, 0.1% (w/v) bovine serum albumin, pH 7.4) and supplemented with 1.1 mM glucose at 37°C for 40 min. Entire cell prepping KRBB (1.1 mmol/L glucose) is then removed and further supplemented with KRBB of which contains at least 2 mM glucose in all conditions (baseline) with compounds of interest used in a dose-dependant manner using n=8 replicate wells. After 2 h of co-incubation with compounds of interest 900 µl was removed from each well and stored until analysed via ELISA at -20°C. Two different GLP-1 ELISAs were used to determine GLP-1 concentration in cell supernatant or *ex-vivo* plasma samples. ELISAs purchased from Millipore (Ontario, Canada) or Merckodia (Uppsala, Sweden) were run according to the manufacturer instructions.

Figure 2. GLP-1 ELISA standard curve

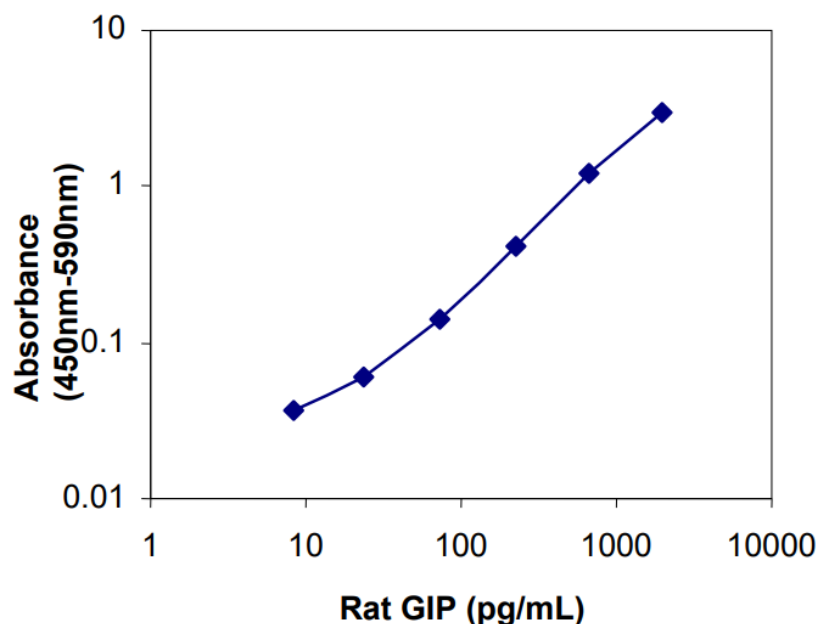


2.2.5. Determination of glucose-dependant insulinotropic polypeptide (GIP) concentration by ELISA

Similar to GLUTag cell cultures, STC-1 cells differentiate by secreting satiety and glucose homeostatic hormones such as cholecystinin (CCK), glucose-dependant insulinotropic polypeptide (GIP), peptide YY (PYY), glucagon-like-peptide 1 (GLP-1) and glucagon-like-

peptide 2 (GLP-2). The experimental procedure was similar to the GLUTag screening procedure. Initially STC-1 cells were seeded onto 24 well plates and incubated with compounds of interest for 2 h and subsequently collected for later analysis. In brief cells were seeded at a cell density of 1.5×10^5 cell/well in 1 ml of DMEM 4500 mg/mL glucose cell culture media and left for 48 h to adhere. On the day of experimentation, media was removed and replaced with 1 ml of priming KRBB (11 mM NaCl, 4.7 mM KCl, 1.28 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.2 mM KH_2PO_4 , 1.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mM NaHCO_3 , 25 mM HEPES, 0.1% (w/v) bovine serum albumin, pH 7.4) and supplemented with 1.1 mM glucose at 37°C for 40 min. Entire cell prepping KRBB (1.1 mmol/L glucose) was then removed and further supplemented with KRBB of which contained 2 mM glucose in all conditions (baseline) with compounds of interest used in a dose dependant manner in n=8 replicates. After 2 h of co-incubation with compounds of interest 900 μl of the supernatant was removed from each well and stored until analysed via ELISA at -20°C. Analysis of GIP from cell supernatant or biological samples using a GIP ELISA (Rat/Mouse) from Millipore (West Lothian, UK) was carried out as instructed by the manufacturer.

Fig 3. Calibration curve for Millipore GIP Rat/Mouse ELISA



2.2.6 Measurement of GLP-1 (7-36_{amide}) degradation during co-incubation with dipeptidyl peptidase 4 (DPP4)

The known degradation of GLP-1 (7-36_{amide}) relies on its interaction with a ubiquitous serine protease hormone, dipeptidyl peptidase-4 (DPP-4). DPP-4 catalyses the cleavage of the penultimate N-terminus amino acid, namely Ala² of the peptide chain. The metabolic effectiveness was rendered obsolete when this cleavage takes place. Active GLP-1 (7-36)_{amide} has several important roles in glucose and satiety regulating homeostasis. GLP-1(7-36)_{amide} in its active form is secreted from intestinal L-cells and has a half-life of approximately 2 min due to the abundance of DPP-4 present in the gut and blood stream. Increasing the active GLP-1 concentration beyond its inactive cleavage molecule GLP-1(9-36) has many advantageous anti-diabetic effects, including increased insulin biosynthesis, increased β -cell survival, glucose dependant reduction of plasma glucose and decreased food intake. Inhibiting the activity of DPP-4 (described in 2.2.8) highlights the potential of enzyme inhibition for the treatment of type 2 diabetes.

2.2.7 Glucose uptake assay via 3T3-L1 transdifferentiated adipocyte cells

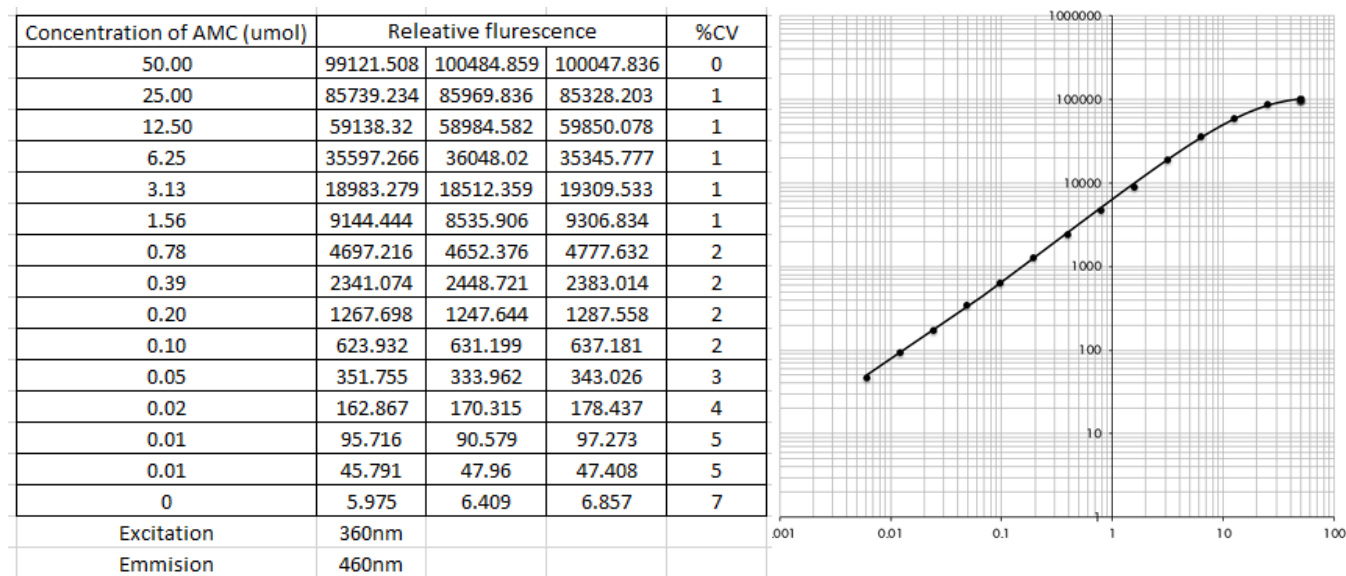
3T3-L1 cells were first cultured in DMEM growth medium until reaching 70% confluence. Cells were split and further seeding into a 96 well plate at a seeding density of 5×10^5 and left overnight to attach. The next day (day 0) growth media was aspirated and fresh pre-warmed grown media containing IBMX (0.5 mM) and dexamethasone (1 μ m). Plates are returned to tissue culture incubator where on day 3, differentiation media was aspirated and fresh growth medium was added containing 10 μ g/mL Insulin. Plates were incubated for a further 3 days, where insulin containing growth media was aspirated and replaced with fresh DMEM culture

media. On day 7 – 10 plates were observed under a microscope where if successful, fully differentiated adipocyte-like cells were obtained for experimentation. Glucose uptake assay was performed directly onto the 96 well plates after aspirating the growth media, replacement of media with KRBB containing necessary test conditions either in the presence of insulin or without insulin. A fluorescently tagged glucose is used to test the glucose uptake efficiency and added to each subsequent well at a fixed concentration.

2.2.8 Direct inhibition of dipeptidyl peptidase 4 (DPP-4) activity *in vitro*

The substrate (H-Gly-Pro-7-amino-4-methyl coumarin, standard (7-Amino-4-methyl coumarin (AMC) and inhibitor control (Diprotin A) were obtained from Bachem Feinchemikalien (Bubendorf, Switzerland). Human recombinant DPP-4 enzyme was obtained from Sigma-Aldrich (Dorset, UK). DPP-4 inhibitory activity was determined by measuring free AMC (7-amino-4-methyl-coumarin) (**Kato *et al.* 1978**) liberated from the fluorogenic substrate Gly-Pro-AMC. Using a Costar, black 96 well plate, 30 μ l of 100 mM TRIS-HCl (pH 7.4) buffer was added into each well. Following this 10 μ l sample or controls was added (n=3) followed by 50 μ l of 200 μ M H-Gly-Pro-AMC. The plate was then incubated at 37°C for 10 min. The reaction was finally started by adding 10 μ l of DPP-4 (8 mU/ml) and incubated for a further 30 min. The plate was read using excitation (360 nm) and emission (460 nm) fluorescence wavelength using a Flex Station 3 (Molecular Devices, CA, USA). A calibration curve was plotted using the standard fluorescent compound 7-amino-4-methylcoumarin from 50 μ M to 0.01 μ M

Fig 4. 7-Amino-4-methylcoumarin standard calibration curve



2.2.9 Assessing the GLP-1 activity in the presence of DPP-4 using RP-HPLC chromatography

Preserving GLP-1 (7-36)amide from degradation to GLP-1 (9-36) in the presence of DPP-4 was achieved by co-incubating with synthetic peptides of marine origin first isolated from *Palmaria palmata*. A 100 mM triethanolamine (TEA) buffer (pH 7.4) was used. In brief, 30 μ l of GLP-1(7-36)amide (10^6 M) was added to a low bind Eppendorf tube (Sigma-Aldrich, Dorset, UK) followed by 30 μ l of sample, DPA control (10^6 M) or blank. Following this 430 μ l of TEA buffer was added. The reaction was started by adding 10 μ l of human DPP-4 (5 mU). Several tubes (n=??) containing the same additions were prepared and subsequently incubated for 0 h, 2 h, 4 h, 8 h and 24 h respectively. The reaction was stopped by adding 50 μ l of 10% TFA. Samples were then stored at -20°C until RP-HPLC analysis.

Analysis of samples was by a RP-HPLC system (Thermo Finnegan, Surveyor) equipped with a UV/VIS detector (Thermo Finnigan Surveyor UV-VIS Plus Detector), 4 port gradient inlet solvent proportioning pump (Thermo Finnigan Surveyor LC Pump), Rheodyne 7125i injector

with a 1000 µl loop, multi-sampler (Thermo Finnigan Surveyor Autosampler) equipped with a 250 µl reservoir and 100 µl column loop. Using a Phenomenex Aeris Peptide C18 (250 x 46 mm / 3.6 µm) solvents (solvents description 2.2.2) starting at 0% solvent B and increased to 30% Solvent B after 10 min then a further increase to 70% solvent B over the next 40 min and finally increasing to 100% solvent B over 5 min and retaining 100% for a further 10 min before returning to starting conditions (100% solvent A). Eluted peaks were collected and stored at -20°C in low bind Eppendorf tubes (Sigma-Aldrich, Dorset, UK) and later analysed using MALDI-TOF MS (PerSeptive Biosystems Voyager-DE Biospectrometer, Hertfordshire, UK).

2.2.10 Compound mass determination using Matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS was performed using a PerSeptive Biosystems Voyager-DE Biospectrometer (Hertfordshire, UK) equipped with a 1 m time-of-flight tube. A 10 mg/ml solution of α -cyano-4-hydroxycinnamic acid (CHCA) (Sigma Aldrich, UK) was prepared in 80% acetonitrile, 20% water with 0.1% trifluoroacetic acid. The matrix was vortexed thoroughly to mix, then spun in a microcentrifuge for 2 min to ensure any undissolved matrix settled to the bottom of the tube; only the supernatant was used. A 10 µL aliquot of each sample was mixed with 10 µL of matrix and a 1.5 µL aliquot pipetted onto a predefined well of a 100-well stainless steel plate. Once the test mixtures had dried on the plate, the plate was inserted into the MALDI-TOF. An internal mass calibration of the instrument was performed prior to sample analysis using Peptide Calibration Mix 2 (LaserBio Labs, Spohia-Antipolis, Cedex, France) containing four individual peptides; Neurotensin, ACTH fragment 18-39, Insulin bovine β -chain oxidised and Insulin bovine with monoisotopic masses of 1672.9176, 2465.1989, 3494.6514 and 5730.6087, respectively.

All sample measurements were collected in linear positive ionisation mode using 50 laser shots/spectrum. The accelerating voltage was maintained at 20,000V, the grid voltage and guide wire voltages were set at 93% and 0.05% respectively, of the accelerating voltage. The nitrogen laser set at 337 nm was directed toward the densest area of the sample / matrix spot and the laser intensity adjusted to obtain the best spectral response. The mass/ charge ratio (m/z) was plotted against relative abundance.

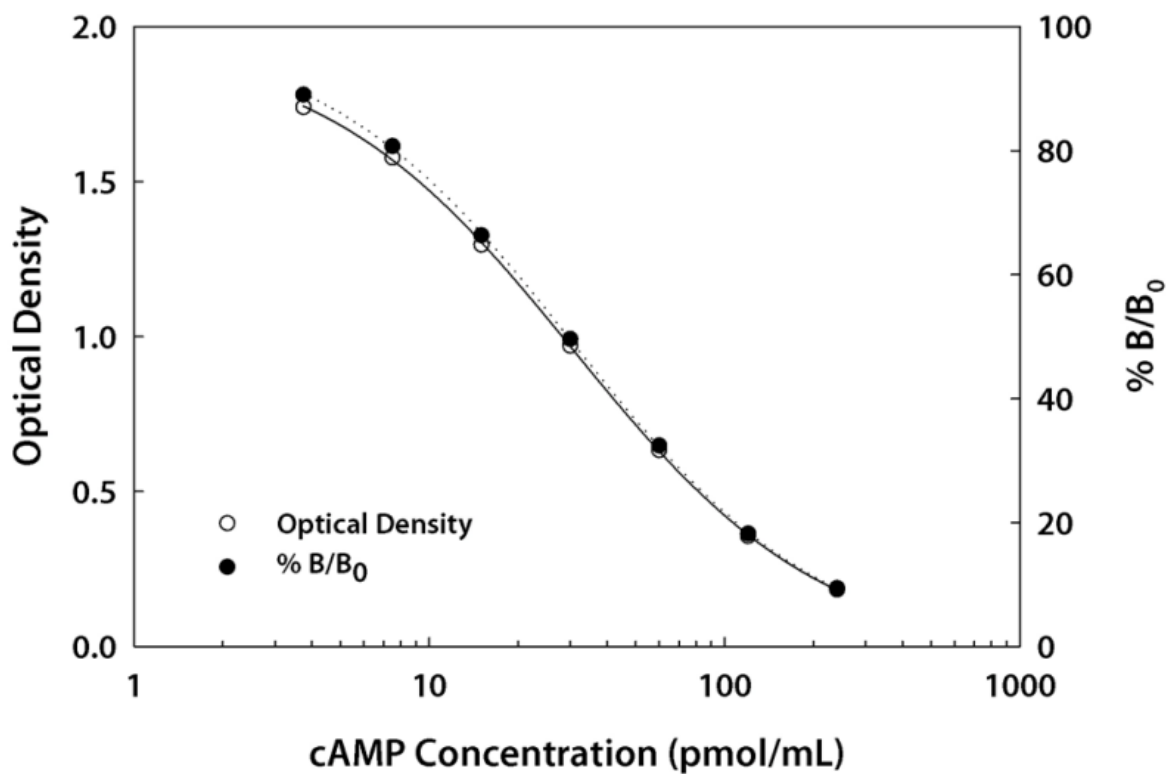
2.2.11 Cell signalling events

2.2.12 Measurement of Adenosine 3,5-cyclic monophosphate (cAMP) production after coincubation with marine protein hydrolysates

The signalling cascade of marine protein hydrolysates on total cAMP release was determined using a commercially available cAMP Parameter assay kit (R&D Systems, Minneapolis, USA). The assay measured cAMP in various samples with cAMP being one of the most important cellular signalling events promoted by the conversion of ATP to cAMP and is imperative to biological functioning. BRIN-BD11 cells were cultured as described in section **2.1.3**. Cells were seeded on 24 well plates at a cell density of 50,000 cells per well with the addition of 1 ml of RPMI-1640 media (10% FBS, 1% PenStrep). Cells were placed into an modified atmosphere incubator (95% air, 5% CO₂) at 37°C for 21 h. Following this the media was removed and 1 ml of prepping KRBB was added (described in 2.2.1) for 1 h. Protein hydrolysates were prepared in working KRBB (described in 2.2.1) with the addition of 5.6 mmol/L glucose and 200 µl of IBMX in the presence of control or control and hydrolysate and incubated for 20 min. Cells were then lysed after removal of supernatant using 100 µl of cell lysis buffer for 30 min.

37°C. Cells were visually inspected to verify cell lysis using a phase contrast microscope (Zeiss, Germany). Samples were stored at -20°C until analysis.

Using the cAMP Parameter assay kit (R&D Systems, Minneapolis, USA) samples were brought to room temperature before being diluted with calibrator diluent RD5-55 from the assay kit. A control serial dilution was prepared from 240 pmol/L to 3.45 pmol/L using the stock 2400 pmol/L standard (Fig 4). The remaining assay was carried out specific to manufacturing protocol instructions. Optical density was read at 450 nm with wavelength correction set to 540 nm using a Flexstation 3 (Molecular devices, CA, USA).



2.2.13 Measurement of membrane potential signalling after co-incubation with marine protein hydrolysates

BRIN-BD11 cells were seeded at a cell density of 90,000 cells per well in Costar, black walled, clear bottom 96 well plates (Appleton-Woods, Birmingham, UK) and incubated for 21 h at 37°C (95% air, 5% CO₂). A fluorometric membrane potential assay was purchased from R&D Systems (Minneapolis, USA). This is a homogenous assay with fast read times. Using a proprietary long wavelength membrane indicator to detect the membrane potential change that is caused by the opening and closing of ion channels. A red indicator was used to minimise interference from compounds or cellular auto-fluorescence. The protocol was run as indicated in the manufacturer guidelines. In brief, 100 µl of working KRBB buffer (described in 2.2.1) supplemented with 5.6 mmol/L glucose was added to each well after removal of media. The plate was then incubated for 10 min. After incubation, the KRBB buffer was left in each well and 100 µl of the FLIPR membrane potential buffer (11 ml of KRBB (5.6 mmol/L) added to R&D premade FLIPR membrane buffer vial) was added to the first column (n=8) of the 96 well plate. The plate was incubated again for 5 min and the 100 µl of the FLIPR buffer was added to the second column of the 96 well plate and incubated again for 5 min. This process was repeated until all columns had received 100 µl of the FLIPR membrane potential buffer and finally transferred to a FlexStation 3 plate reader (Molecular devices, CA, USA) which had its plate compartment pre-warmed to 37°C. Using an excitation and emission wavelength (Ex 360 nm, Em 460 nm), the software had been programmed to read the first column for 5 minutes after it added 10 µl of sample to each well. After 5 min the plate reader automatically added the same samples to the next column and read for a further 5 min. This

was continued until all columns had been aliquoted with sample and read for 5 min. The data was combined and later analysed using Graphpad™ Prism 5 statistical software.

2.2.14 Measurement of Intracellular calcium (Ca²⁺) signalling after co-incubation with marine protein hydrolysates

The FLIPR calcium assay (Molecular Devices, CA, USA) measures changes in intracellular calcium only via its proprietary fluorescent dye. This represents a robust, sensitive assay for use with a broad range of compounds including food derived and pharmaceutical. The kit delivers a pre-optimized, homogenous, fluorescent-based formulation of which is vital for GPCR and ion based channel activation. BRIN-BD11 cells were seeded at a cell density of 90,000 – 100,000 cells per well in Costar, black walled, clear bottom 96 well plates (Appleton-Woods, Birmingham, UK) and incubated for 21 hat 37°C (95% O², 5% CO₂). A fluorometric membrane potential assay was purchased from R&D Systems (Minneapolis, USA). This is a homogenous assay with fast read times. Using a proprietary long wavelength membrane indicator to detect the membrane potential change that is caused by the opening and closing of ion channels.

The protocol was run as indicated by the manufacturer guidelines. In brief, 100 µl of working KRBB buffer (described in 2.2.1) supplemented with 5.6 mM glucose was added to each well after removal of media. The plate was then incubated for 10 min. After incubation, the KRBB buffer was left in each well and 100 µl of the FLIPR membrane potential buffer (11 ml of KRBB supplemented further with 10 mM calcium chloride (CaCl₂) and 100 X probenecid) added to R&D premade FLIPR membrane buffer vial) was added to the first column (n=8) of the 96 well plate. The plate was incubated again for 5 min and the 100 µl of the FLIPR buffer was added

to the second column of the 96 well plate and incubated again for 5 min. This process was repeated until all columns had received 100 µl of the FLIPR membrane potential buffer and finally transferred to a FlexStation 3 plate reader (Molecular devices, CA, USA) of which had its plate compartment pre-warmed to 37°C. Using an excitation and emission wavelength (Ex 315 nm, Em 400 nm), the software was programmed to read the first column for 5 min after it added 10 µl of sample to each well. After 5 min the plate reader automatically added the same samples to the next column and read for a further 5 min. This was continued until all columns had been aliquoted with sample and read for 5 min. The data was combined and later analysed using Graphpad™ Prism 5 statistical software.

2.2.15 Cellular viability assay using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide).

Investigating cellular redox reactions is used a tool to identify potential cellular stability, toxicity or proliferation in various cell lines when challenged with various molecules. While the overall reaction converts a water soluble MTT compound to an insoluble formazan, the rate at which this is carried out can be accurately used to investigate cellular functionality and potential cytotoxicity issues that may arise from a compound of interest.

Experimental conditions require cells to be seeded at a cell density of $10 - 100 \times 10^4$. Cells were left to adhere overnight in culture medium suited for optimal growth. On day of experimentation, test conditions were prepared in KRBB buffer with various concentrations of glucose which match the test conditions seen in the acute secretory activity study (refer to section 2.1.2/3 for test condition preparation).

On completion of compound co-incubation with KRBB, this was removed and cells were carefully washed with HBSS. Furthermore 100 µl of growth media was added back to each well of the 96 well plate and further supplemented with 20 µl of MTT solution (5 mg/ml stock).

The 96 well plate was then incubated for 2 – 4 hours (dependent on cell density) in a modified atmosphere (95% air, 5% CO₂) tissue culture incubator at 37°C.

The plates were visually inspected with MTT growth media aspirated and washed for a final time with 100 µl of HBSS. On completion of the final wash, 100 µl of DMSO was added, which dissolves the insoluble formazan crystals. The 96 well plate is placed onto an orbital shaker at RT for 5 min. Finally using a spectrophotometer, the plate was read at absorbance 570 nm with 630 nm correction. Results were then generated and analysed using Graphpad Prism 5.

2.2.16 Identifying cellular toxicity using cytotox 96 non-radioactive cytotoxicity assay

Lactate dehydrogenase (LDH) is a relatively stable and expressed intracellular enzyme which catalyses the conversion of lactate to pyruvic acid and vice versa (interconversion). LDH acts upon hydrides (single hydrogen accepting molecule) allowing NAD⁺ to convert to NADH and back again. This vital cellular respiratory activity makes LDH detection a valuable tool for cellular viability and toxicity. As LDH is an intracellular metabolite, the detection of this enzyme on the outside of the cell is a common marker of cellular injury and damage

Using the cytotox 96 non-radioactive cytotoxicity assay (Promega, UK), cellular investigation was carried out using cultured cell lines at a cell density of 150×10^5 . Experimentation was carried out exactly as described in 2.1.2 with a final step of testing 50 µl of collected supernatant aliquoted into 96 well plates using test conditions (n=3) for released LDH concentration. Using the assay kit, 50 µl of cell supernatant was mixed with 50 µl of Promega LDH substrate solution and incubated at RT protected from UV light for 30 min. Once complete, 50 µl of stop solution (NaOH) was added and finally read at absorbance 595 nm

using a spectrophotometer 96 well plate reader. Results are then generated and analysed using Graphpad Prism 5.

2.3. Animal models

2.3.1 Normal mice (NIH Swiss)

NIH Swiss mice (Harlan UK Ltd., Blackthorne, UK) derived from a nucleus colony obtained from the National Institute of Health, Bethesda, Maryland were used for acute glucose lowering experiments. Prior to experimentation mice were acclimatized for 1 week on arrival on a standard rodent diet (10% fat, 30% protein, 60% carbohydrate; percentage of total energy 12.99 kJ/g; Teklad Natural diet for rodents) (Envigo, Blackthorn, UK).

Mice were housed in an air conditioned room maintained within a narrow temperature range of $22 \pm 2^{\circ}\text{C}$ with a 12 h light: 12 h dark cycle. Mice were used as a model of euglycemic. Mice were grouped depending on their body weight and non-fasting blood glucose concentration. Drinking water and standard rodent diet was freely available. All animal studies were performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

2.3.2 Genetically-induced obese-diabetic (*Ob/Ob*) mice (B6.Cg-Lepob/J)

Ob/Ob mice carry the homozygous gene for spontaneous mutation of the *Lep^{ob}* autosomal recessive chromosome 6 (Harris *et al.* 1998). Subsequently this model exhibits rapid onset of obesity (4 – 6 weeks) and obesogenic conditions commonly studied in metabolic research such as hyperphagia, glucose intolerance, elevated plasma insulin levels including hyperglycaemia making this rodent an good model for type 2 diabetes and potential

treatment of glucose lowering compounds and dietary related metabolic changes (Bailey and Flatt, 1982). Mice (10-12 week old) were ordered from Envigo, Blackthorn, UK.

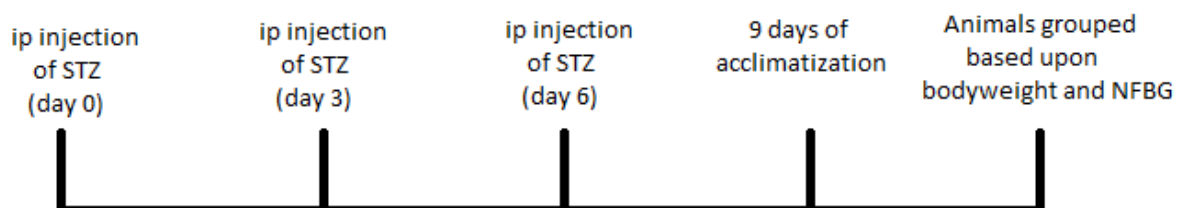
Mice were housed in an air conditioned room maintained within a narrow temperature range of $22 \pm 2^{\circ}\text{C}$ with a 12 h light: 12 h dark cycle. Mice were used as a model of insulin resistant type 2 diabetes Mice were grouped depending on their weight and non-fasting blood glucose concentration. Drinking water and standard rodent diet was freely available. All animal studies were performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

2.3.3 Multiple low-dose Streptozotocin induced diabetes model

A large one-off dose of Streptozotocin (STZ) in fasted mice are typically used for the study of non-insulin resistant, insulin-dependent type 1 diabetes (Wu & Huan, 2008). Streptozotocin has a toxicity affinity toward the pancreatic β -cell causing a cascade of downstream effects including direct DNA methylation and eventual cell death via its high affinity for the GLUT2 protein receptor, of which are in abundance on β -cells (Bolzán & Bianchi, 2002). Streptozotocin has no cross reactivity for other GLUT protein receptors explaining its potency for pancreatic β -cells. Doses used for type 1 diabetes are typically 165 – 220 mg/kg bodyweight in mice and 40 – 50 mg/kg in rats, however lower doses have been used successfully to partly inhibit insulin secretion via reduced cellular damage of the pancreatic β -cells (McEvoy *et al.* 1984; Deeds *et al.* 2011). This in-turn causes moderately elevated blood glucose concentrations. Insulin production was still evident in this model without the insulin resistance seen in other obesity derived diabetes rodent models.

HsD:Ola T0 mice (10 weeks old) were obtained from Envigo, Blackthorn, UK. Mice were housed in an air conditioned room maintained within a narrow temperature range of $22 \pm 2^{\circ}\text{C}$ with a 12 h light: 12 h dark cycle. Mice were grouped depending on their body weight and non-fasting blood glucose concentration. Drinking water and standard rodent diet was freely available. All animal studies were performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

Animals were injected with Streptozotocin (40 mg/kg bodyweight), which was prepared in a citrate buffer containing sodium citrate dehydrate (0.08 M) and citric acid (0.018 M) and pH to 4.5 with 1 M HCl. Drinking water was changed for sucrose water to reduce the potential overnight hypoglycaemia commonly seen with STZ injections. On the third day after the injection blood glucose was obtained from a tail bleed sample using a Bayer Contour glucometer (Bayer, Leverkusen, Germany). This process was repeated twice more for a total of 3 x 40 mg/kg bodyweight injections. Animals were given 9 days of acclimatization subsequent hyperphagia, polydipsia and hyperglycaemia to develop before they were grouped according to non-fasting blood glucose concentration and weight.



2.3.4 Satiety and appetite study in 3 h fed trained animals

Metabolic hormone homeostasis is controlled within narrow ranges in healthy individuals, however these regulatory mechanisms can be altered in conditions of severe obesity or in subjects with type 2 diabetes. Food intake and satiety hormones such as leptin and ghrelin present a diminished biological triggers when obesity, hyperglycaemia and hyperinsulimia are present (Klok *et al.* 2007). Using healthy animals which have controlled satiety after oral ingestion of nutrients can be used for assessing appetite for compounds that may promote satiety. Several hormones have been shown to play a role on promoting alterations to food intake such as leptin, ghrelin and more recently peptide YY (PYY 3-36) (Batterham & Bloom, 2003) and cholecystinin (CCK-8) (Little *et al.* 2005). When PYY or CCK is administered via intraperitoneal (ip) injection, food intake is reduced whereas the opposite is observed with ghrelin.

Hsd:Ola T0 mice (8 weeks old) were obtained from Envigo, Blackthorn, UK. Mice were housed in an air conditioned room maintained within a narrow temperature range of $22 \pm 2^{\circ}\text{C}$ with a 12 h light: 12 h dark cycle. Drinking water and standard rodent diet was freely. All animal studies were performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

On arrival animals had access to food 24 h per day for 1 week. This was reduced to 10 h of food availability on week 2 with further reduction to 6 h by week 3. Finally, on week 4 and for the remaining duration of the animal satiety study, food availability was 3 hours per day from 10.00 h to 13.00 h

2.4 Acute animal studies

2.4.1 Establishing the acute oral dose required for glucose lowering effect of marine protein hydrolysates

A working hydrolysate dose was initially established using several interchangeable dosing approaches depending on the protein hydrolysate used. An initial hydrolysate dose of 100 mg/kg bodyweight was used in experiments and further titrated to 50 mg/kg and 25 mg/kg until effect, if any was evident. The animal model used were NIH Swiss mice (**section 2.3.1**). Each group (n=8) were fasted for 8 h prior to experimentation and housed as described in 2.3.1. Food was withheld during experimentation however access to water was available. Prior to compound administration a fasting tail blood sample was analysed for glucose concentration using a handheld glucometer (Bayer Contour, Leverkusen, Germany). A fixed dose (25 – 100 mg/kg bodyweight) of hydrolysate was then mixed with a dose of glucose (18.8 mmol/kg bodyweight) and administered orally via gavage. The effectiveness of each hydrolysate was then established by further testing blood glucose concentration at various timepoints up to 2 h (15, 30, 60, 90 and 120 min). After experiments were complete mice were given access to food and given a 72 hour washout period before any further experimentation was carried out on the same group of animals.

2.4.2 Establishing the acute duration of biological action of marine protein hydrolysates

Hydrolysates that were identified as having an oral glucose lowering effect were then carried onto further investigation using a delayed glucose tolerance test. This was investigated via

oral compound administration during fasting, followed by glucose (18.8 mmol/L) administration either 4, 8 or 12 h later to identify if a lasting glucose lowering effect was evident from the hydrolysate of choice. The glucose lowering dose of the hydrolysate was previously identified, mice were then fasted for 4 h and then administered a dose of hydrolysate depending on its glucose lowering effect. Animals were given access to only water and at 4, 8 or 12 h the animals were then given glucose (18.8 mmol/L) via oral gavage. Tail blood glucose concentrations were recorded using a handheld glucometer (Bayer Contour, Leverkusen, Germany). Similar to acute animal study, blood glucose concentration were established prior (0 min) and after oral glucose administration (15, 30, 60, 90 and 120 min). Animals were then given access to food and given 72 h washout before further experiments could commence.

2.4.3 Assessing the anti-diabetic efficacy of marine protein hydrolysates in obese diabetic *ob/ob* mice after chronic twice daily oral administration

The acute and delayed glucose lowering effect of various marine protein hydrolysates were previously established using healthy NIH Swiss mice with homeostatic control of blood glucose concentrations. Using an animal model described in 2.3.2, 8 week old (n=32) *ob/ob* mice were given several weeks to fully establish markers of spontaneous type 2 diabetes, including obesity, hyperglycaemia, hyperphagia and polydipsia before being grouped according to bodyweight and non-fasting blood glucose concentration. Animals were grouped and assigned either a MPH compound or control. All animal groups were given twice daily oral saline to acclimatize for 3 days (day -3 to 0) and the assigned treatment started twice daily (09.00 h am & 17.00 h) from day 0 to day 27 after which some terminal analysis commenced. Mice were maintained on the twice daily treatment regime from day 27 until

the completion of the study (day 30). During the study duration (30 day total) animals were assessed every third day with blood glucose concentration, weight and food intake recorded alongside collection of blood plasma for future analysis. Collected blood plasma was centrifuged at 13,000 rpm for 5 min and stored in low-bind Eppendorf tubes at -20°C until analysed.

2.4.2 Terminal parameters assessed in *ob/ob* animals

2.4.3 Glucose tolerance tests (intraperitoneal and oral)

Blood samples were measured from a minor tail vein bleed in fasted (8 h) *ob/ob* mice. Blood glucose was measured using a handheld glucometer (Bayer Contour, Leverkusen, Germany), prior (t=0) to administering a glucose challenge (18.8 mmol/kg/bodyweight). Once administered, blood glucose was further analysed at 15, 30, 60, 90 and 120 min. At each timepoint a quantity of blood (approx. 50-80 µl) was collected into fluoride coated microcentrifuge tubes (Starstedt, Mimbrecht, Germany) and immediately centrifuged at 13,000 rpm for 5 min and stored frozen at -20°C in a fresh Eppendorf tube prior to insulin determination.. Plasma insulin concentration was determined by insulin radioimmunoassay (described in 2.2.3).

2.4.4 Insulin sensitivity test

Blood glucose concentrations of animals were measured from a minor tail vein bleed in non-fasted mice using a handheld glucometer (Bayer Contour, Leverkusen, Germany). Blood glucose concentrations were measured prior to (t=0) and at 30 and 60 min post injection

following ip administration of bovine insulin as 25 U/kg bodyweight (normal NIH Swiss / HsdOlaT0 / C57Blk) or 50 U/kg bodyweight (*ob/ob* mice).

2.4.5 Measurement of indirect calorimetry

Mice were housed individually in a Complete Laboratory Animal Monitoring System (CLAMS) metabolic chambers (Columbus Instruments, Columbus, Ohio, USA). Consumption of O₂ and production of CO₂ was measured for 30 sec at 15 min intervals for a total of 20 h. Animals were housed within the CLAMS apparatus for 24 h prior to analysis to reduce anxiety related result bias. Respiratory exchange ratio (RER) was calculated by dividing VCO₂ by VO₂. Energy expenditure was calculated using RER with the following equation $(3.815 + 1.232 \times \text{RER}) \times \text{VO}_2$. Ambulatory locomotor activity of each mouse was measured simultaneously using optimal beams (Opto M3, Columbus Instruments, Columbus, Ohio, USA). A breakage of photo-beams (X, Y and Z directional plane) was measured as physical movement recorded every min for a 20 h period.

2.4.6 Tissue excision

On completion of all terminal experiments mice were fasted (4 h) and sacrificed. Mice were placed unconscious via oral inhalation of a general anaesthetic (Isoflurane) and euthanized via cervical dislocation. The pancreas from each animal was excised removing any extraneous material. The pancreas was sectioned in half from the pancreas head to tail with half being wrapped in aluminium foil and snap frozen in liquid nitrogen and later stored at -70°C and the remaining half being placed into tissue fixative solution (4% paraformaldehyde) for later use in tissue processing and immunohistochemistry.

2.4.7 Pancreatic hormone content

Thawed tissue was rinsed in cold PBS before being weighed and transferred to a bijou where 2 ml of ice cold acid ethanol (1.5% (v/v) HCL, 75% (v/v) ethanol, 23.5% (v/v) H₂O) was added. The bijou was then placed on ice for 30 min before being homogenized for 30 sec. The bijou was blitzed using a high-speed Homogenizer for 30 sec intervals for a total of 2 min per pancreas and placed back onto ice. The homogenate-containing bijou was kept on ice until centrifuged at 4000 rpm for 20 min with the resulting supernatant transferred to a fresh tube. Total volume was then increased by addition of 5 ml of TRIS-HCl (pH 7.4) and placed into a speed vac concentrator (45°C for 4 h) until all liquid was evaporated. The remaining sample was reconstituted in 500 µl of TRIS-HCl (pH 7.4) and stored in 1.5 ml polypropylene Eppendorf tubes at -80°C until analysis.

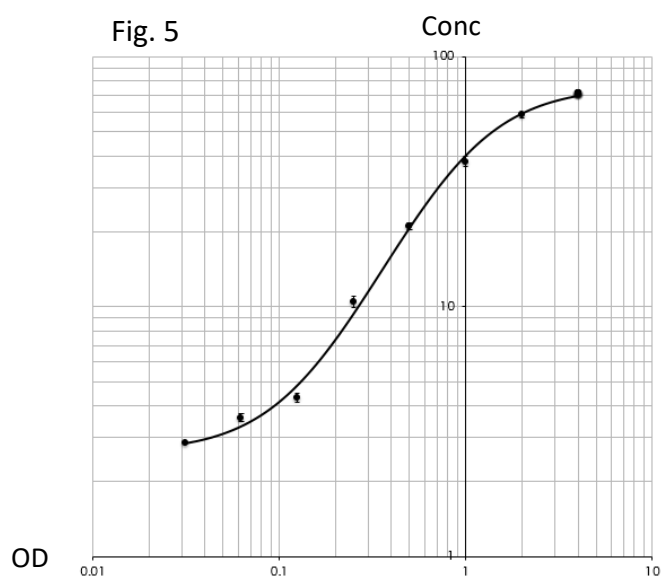
2.4.8 Assessment of pancreatic insulin and glucagon content

The pancreatic homogenate was diluted to a range of concentrations (1:100, 1:200, 1:500 and 1:1000) using stock RIA buffer prepared in section 2.2.2 and tested for insulin content using the RIA described in section 2.2.3. Pancreatic glucagon content was assessed using a chemiluminescent sandwich ELISA (Millipore, Ontario, Canada) and run according to manufacturer instruction. Insulin and glucagon content were then calculated and further compared to total pancreatic protein content using the Bradford assay method for determining total protein content of the original sample.

2.4.9 Total pancreatic protein content

Insulin RIA and glucagon ELISA were used to determine total pancreatic hormone content. In order to quantify detected hormone percentage it was compared to total pancreatic protein concentration from tissue homogenate described in 2.4.7. The Bradford assay is a robust assay for its sensitivity and linear protein quantification using diluted samples. The assay was run at room temperature and results were generated using a microplate reader. Protein standards were prepared using a fixed concentration of bovine serum albumin (BSA) and diluted in a serial dilution. Initially 10 μ l of pancreatic homogenate was diluted with deionized H₂O (1:10). The diluted sample was then pipetted onto a 96 well plate in duplicate (10 μ l per well) with the addition of 250 μ l of Bradford reagent added (Sigma-Aldrich, Dorset UK). The plate was then placed on an orbital shaker at 300 rpm for 5 min at room temperature. Finally the plate was read at wavelength 595 nm using a microplate reader (Flex Station 3, Molecular devices, CA, USA). Bradford reagent only was used as the blank control and was used to subtract the baseline absorbance. Results were plotted against the absorbance of the BSA calibration curve detailed in Fig 5.

Fig. 5



Control BSA concentration used (mg/ml)	Concentration given from standard	
2	1.942993537	1.997109257
1.5	1.368010583	1.306675103
1.125	1.066143515	1.014222876
0.84375	0.821293029	0.769505098
0.6328125	0.599225069	0.57879704
0.474609375	0.43763023	0.41146972
0.355957031	0.317086195	0.319783487
0.266967773	0.223337896	0.239100276

2.4.10 Assessment of terminal plasma lipid profile

Terminal blood plasma lipid profile was determined using an I-Lab 650 clinical chemistry system (Instrumentation Laboratory, Warrington, UK). The analysis consisted of assessing total triglycerides, total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) concentration. Reagents for triglyceride analysis were obtained from Instrumentation Laboratory (Warrington, UK) and reagent for LDL cholesterol were obtained from Randox Healthcare (Randox, Co, Antrim, NI).

2.4.1 Assessing the anti-diabetic effect of marine protein hydrolysates in Streptozotocin diabetes induced mice after twice daily oral administration

Using an animal model described in 2.3.3, 10 week old (n=32) *HsdOla:TO* mice were administered several low doses of Streptozotocin (40 mg/kg bodyweight x 3 over 6 days and monitored daily for 9 days total, (day 0, 3, 6 & 9), which causes β -cell damage with partial insulin secretion still observable. Animals were given 9 further days to acclimatize after the last STZ injection. Animals were monitored for signs of hyperglycaemia, weight loss, hyperphagia and polydipsia. Once the acclimatization period ended mice were grouped (n=7-8) according to non-fasting blood glucose concentration and bodyweight. Each group was assigned a treatment and prior to treatment commencing animals were further acclimatized for 6 days (study day -6 to day -1) with twice daily oral saline (0.9% NaCl) gavage (09.00 h and 17.00 h) to mimic the treatment which started on study day 0. During the study duration (18 day total) animals were assessed every third day with blood glucose concentration, body weight and food intake recorded alongside collection of blood plasma for future analysis.

Collected blood plasma was centrifuged at 13,000 rpm for 5 min and stored in low-bind Eppendorf tubes at -20°C until analysed.

2.4.2 Assessment of food intake in 3 hour trained feeding HsdOla:TO mice

Hsd:Ola:TO mice (8 weeks old) were obtained from Envigo, Blackthorn, UK. Mice were housed in an air conditioned room maintained within a narrow range of $22 \pm 2^\circ\text{C}$ with a 12 h light: 12 h dark cycle. Drinking water and standard rodent diet was freely available on arrival. Mice were trained to eat for 3 h per day as described in section 2.3.4. Before animals were given access to food for 3 h, food averaging 10 g was weighed. Animals (n=8) were administered a chosen compound by oral gavage dissolved in saline or a saline vehicle (0.9% NaCl) with food presented and weighed subsequently every 30 min up to 3 h. Food would then be removed until the following day. Food intake was calculated and compared to the saline only (0.9% NaCl) control group.

2.5 Human study

2.5.1 Materials

Rapilose™ OGTT solution (orange flavour, WHO recommended 75 g of glucose) were purchased from Galen Pharmaceuticals (Portadown, Co. Armagh, NI). Blood collection tubes were purchased from BD Biosciences (Oxford, UK) including serine protease inhibitors for the preservation of GLP-1 (P800 clear cap, 2 ml), fluoride oxalate tubes for the preservation of plasma glucose (grey cap, 2 ml) and anti-coagulation tubes for the preservation of whole blood plasma (lavender cap, 6 ml). Plasma collected was stored in Low-Bind© Eppendorf tubes purchased from Sigma-Aldrich (Poole, Dorset, UK). Hydrolysate (Boarfish) was obtained from University of Limerick following processing described in section 2.1. A tomato based chilli flavoured soup was produced and supplied by Ulster Catering College (Belfast, NI) and stored at -20°C until required then heated to 90°C and cooled before serving.

2.5.2 Rescreening acute antidiabetic effect of Boarfish (*Capros Aper*) after scaling up of hydrolysate production

Production of boarfish protein hydrolysate generated and sent to Ulster University from University of Limerick (as **described in section 2.1 / semi-pilot scale**) was scaled up and required subsequent re-screening for retention of bioactivity in cell cultures (as **described in section 2.2.1**) and in acute *in vivo* setting (**described in section 2.4.1**).

2.5.3 Recruitment of study participants

Ethical approval was sought via Ulster University Research Governance of research involving human participants (UREC). Approval for research with 20 participants was granted on 20th September 2016 (**See appendix ?**). The study required 20 participants of which were recruited

from within the University campus or neighbouring Causeway coast area. A mixed cohort of males and females (10 male 10 female) with body mass index below 25 kg/m² were screened using anthropometric analysis before being confirmed as eligible for participation on the study.

2.5.4 Acute effect of Boarfish (*Capros aper*) hydrolysate on markers of obesity, diabetes and satiety in healthy individuals

The study was designed as a double-blind placebo controlled crossover trial that required two separate visits to the Ulster University, Human Intervention Study Unit (HISU) at Coleraine. Participants were randomly assigned to either placebo or control by a member of staff who was independent from the research team. Participants were required to fast from 22.00 h (if they were required to attend a 07.30 h session) or 23.00 h (if a 08.30 h session was attended). On arrival, a fasting blood sample was obtained via cannulation of the medial cubital vein on the non-dominant arm. A short questionnaire (visual analogue scale, VAS) was completed on feelings of hunger and current taste perception. The participant was then asked to consume a tomato based chilli soup (60 ml) which was or was not supplemented with 3.5 g of boarfish protein hydrolysate. Following this the participant was then asked to consume the RapiLOSE™ OGTT solution (300 ml) within 5 - 10 min. Blood samples were subsequently taken at set timepoints after completion of the OGTT solution (15, 30, 60, 90, 120, & 180 min) with further VAS on hunger and satiety completed by the participant at each blood collection timepoint.

2.5.5 Collection of blood plasma for later analysis

Blood collected was processed immediately on withdrawal from each participant. Vials were first placed onto an orbital blood roller for 5 min before centrifugation. Vials were centrifuged at the manufacturers recommended instructions (P800 tube at 2000 g for 10 min, grey and lavender vials 3000g for 15 min). Separated plasma were finally labelled and aliquoted into smaller volumes in low-bind Eppendorf tubes and stored at -80°C until further use.

2.5.6 Plasma analysis of glucose homeostasis and satiety hormones

The study outcome aimed to analyse acute short-term markers that may indicate oral food derived antidiabetic compounds within the boarfish protein hydrolysate. Initially blood glucose concentration from 0 min to 180 min was measured using an I-Lab 650 clinical chemistry system (Instrumentation Laboratory, Warrington, UK). Blood plasma collected via BD P800 serine protease inhibitor blood collection tubes were analysed for glucose homeostasis metabolites and metabolites of satiety using Meso Scale Discovery (MSD) 4-plex, GLP-1 (7-36)amide, insulin, glucagon and leptin (Kit - K15174C, Meso Scale Discovery, Maryland, USA). Meso Scale Discovery uses a sandwich immunoassay with proprietary MSD SULFO-TAG™ technology allowing each labelled hormone to bind to their specific working surface electrode. Any unbound surface electrodes are blocked by the use of bovine serum albumin (MSD Blocker) and when read using the MSD Sector Imager, this correlates surface electrochemiluminescence to plasma metabolite concentration via linear calibration curves. Accuracy was assessed using a single pooled plasma sample and plate variance reported as sample coefficient of variance (CV%) between each metabolite assessed.

2.5.7 Analysis of self-reported satiety and food preference using visual analogue scales

Participants were required to attend both sessions fasted with perceived hunger and food preference assessed using visual analogue scales at each blood collection time point, prior and post consumption of the assigned compound. Each visual analogue scale was rated from 0 to 10, with 0 as the lowest score of perceived hunger and 10 the highest score of perceived hunger. Similarly, food preference during the study was investigated determining if food preference was altered with the intake of each assigned compound. Each VAS was measured to the closest mm and scores were entered into IBM™ SPSS© statistical software and calculated using Students t-test and ANOVA.

Chapter 3

Acute screening of marine protein hydrolysates using
diabetes specific *in vitro* bioassays

3.1 Introduction

Protein

Proteins are nitrogen-containing compounds consisting of amino acids joined together by peptide or covalent amide bonds. For aspects of life, proteins serve as vital structural compounds in muscle and tissue within all living organisms (Hoffman & Falvo, 2004). In addition to making up integral structural components of an organism, proteins act as physiological messengers, signalling molecules, enzymes and hormones that play imperative roles for biological functioning (Walther & Sieber, 2011). The energy that can be supplied via proteins is the same as carbohydrates however only when the protein is degraded to its simplest form, an amino acid. Unlike its caloric rival glucose, protein is regulated within tight requirement basis with protein being required on a day-to-day basis. This information leaves protein, one of the 3 main macronutrients, ruled out as a direct cause of obesity during protein imbalance, however increased protein intake without the requirement may affect fat balance (Galgani & Ravussin, 2008). The makeup of a protein consists of a large number of amino acids in series ($\geq 80-400+$), which can contain any one of the 21 amino acids identified that are needed for human growth, repair and metabolism. The adult human body can synthesize 11 of the 20 amino acids under normal physiological conditions termed non-essential, however, requirements must be met from exogenous sources for the remaining 9 essential amino acids required. Additionally the non-essential amino acids can be further categorized into conditionally essential when higher than average requirements are needed which can be seen in a disease state or in an exercising individual (Lacey & Wilmore, 1990)

Intake of protein at above recommended levels is widely accepted for those with athletic goals or competing in resistance and endurance sports (Phillips & Van Loon, 2011). Protein intake levels typically exceed the recommended daily amount in a healthy, active individual. Recent trends in weight loss have led to a marked increase in protein intake globally, the recommendations of 0.8 g/kg BW/d is contrived in individuals that have renal impairment and as such should closely monitor protein intake (Martin et al, 2005). The renal impairment, the ability of the kidneys to remove the amino acid metabolites and urea waste from the blood is hindered and as such accumulates.

There is concern over increased protein intake and the promotion of renal impairment in otherwise healthy individuals, however, with this noted the question relies on sufficient evidence provided to assess such a statement. Protein intake is a reported modulator of renal function and as such has spawned the ongoing controversy over compensatory renal hypertrophy and thus the changes seen in renal function (Calderon et al, 2004). At this moment, elevated intakes of protein are not cause for global concern with several studies providing beneficial study outcomes of increased protein intake, mainly the offset of other macronutrients such as carbohydrates, cause increased secretion of satiety hormones (Leptin, GIP and GLP-1) (Karamanlis et al, 2007), while having the opposite effect on the orexigenic hormone ghrelin (Leidy et al, 2007) with acute improvement in glycaemic profile in non-insulin dependent type 2 diabetes patients (Pomerleau et al, 1993).

The incorporation of protein with carbohydrates has led to researchers investigating the link between glucose excursions or decreased post-prandial glucose elevation. In patients with type 1 diabetes, high protein dietary incorporation increased glucose excursion however had a protective effect lowering hypoglycaemic events (Smart et al, 2013). Using macronutrient comparisons, on a gram to gram basis, protein has the ability to elevate post-prandial glucose concentration as efficiently as carbohydrates alone. This was seen in high-protein low carbohydrate diets in untreated type 2 diabetic patients, overall glycaemia was chronically improved suggesting enhanced gluconeogenesis and reduced fasting glucose concentration due to carbohydrate reduction with insulin sensitivity enhancement (Gannon & Nuttal, 2004). With this noted, proteins combined with carbohydrates displayed attenuated glucose elevation when observed and overall acute glucose lowering effect having 2 to 3 fold greater effect than fat supplementation (Moghaddam et al, 2006). The mechanism of this is now understood as the post-prandial incretin effect accompanied with reduced gastric emptying. The intestinal nutrient sensing cells are also involved via secretion of potent insulin secreting hormones of which will be discussed later in greater detail (Karamanlis et al, 2007).

The source and type of protein affects the displayed therapeutic effect, otherwise described in layman's as fast or slow proteins, of which display various effects on blood glucose spikes in both healthy individuals or those mild to moderate glucose dysregulation. The digestion rate and appearance of amino acids within the blood stream describe the characteristics of the protein (Deutz & Wolfe, 2013). The impact of post-prandial glycaemia

on health and disease prevention related to metabolic syndrome and diabetes is an area of wide academic interest. Chronically increased nutritional intake in relation to hyperinsulinemia and lipidaemia is implicated in the development of obesity, cardiovascular disease and type 2 diabetes mellitus (Ley et al, 2014). Carbohydrates are the most important macronutrient within the human diet as a fuel source, however interest in protein, protein hydrolysates and its peptide and amino acid degradation products have directed research to rethink the overall goal of high protein intake for attenuation of health and disease states especially those related to glucose homeostasis and diabetes (Manders et al, 2006; Van loon et al, 2003; Clemmensen et al, 2013).

Common protein sources

Exogenous proteins are available from a wide array of dietary sources. Typically the human diet consists primarily of protein from animal and plant origin (Bilsborough & Mann, 2006). More recently dietary patterns and the incorporation of protein from supplemental sources have become increasingly popular (Wolfe, 2000). Nitrogen based supplements take full advantage of industry by-products, such as whey protein during cheese manufacturing. By-products are often resold and processed, powdered and flavoured for addition to food matrixes or high quality food replacement or addition supplements for nutritional improvement, sports and exercise use and bioengineering (Kapoor et al, 2017). Dietary incorporation of complete proteins, those of which contain all the essential and non-essential amino acids is accomplished easily from animal sources whereas proteins of plant origin lack several amino acids that must be obtained from several sources, i.e. a vegetarian can obtain all amino acids more efficiently via dietary incorporation of several different plant sources rather than relying on a single biological source of protein (Young & Pellett, 1994)

Dairy derived protein types

In recent years data from epidemiological studies has shown that dairy consumption is associated with a decrease prevalence of metabolic related disorders with experimental studies pointing towards dairy protein as holding the key to this success (McGreggor and Poppitt, 2013). Increasing dietary intake of dairy derived proteins are now widely available from many sources of dairy. Whey is described as the transient liquid part of milk that is left over following the coagulation and curd removal in cheese making abundant in beta-

lactoglobulin and alpha-lactalbumin. Proteins within the liquid can be separated using various techniques and purified to the desired protein concentration afterwards. Whey is described as one of the 2 main milk derived proteins with whey accounting for approximately 20-25% of total protein content with casein accounting for the remainder. Milk protein sources provide a large quantity of readily available amino acids of which makes whey protein a valuable product worldwide. Nowadays It's not surprising that the idea surrounding milk and milk products having a beneficial metabolic effect as its complex in nature, containing all the vital nutritional components to sustain and support life and nutritional development (Pasin & Comerford, 2015).

The processing of whey has several advantages, whey powder with minimal processing can be used as a food additive seen in snacks, confectionary and bakery items. Its sensory characteristics vary which can be used to alter the taste of a product making it a valued product in food manufacturing. Concentrate whey however involved hands-on processing of which aims to remove unwanted lactose, lipids and various minerals. This intermediate processing step between whey and whey isolate is favoured in sports due to its biologically active components, micronutrient and trace element profile (Geiser, 2003). Lastly whey isolate involved rigorous processing and yields the highest protein content of all whey variants described, however with intensive processing denaturation of the protein structure removing peptide bonds and lowering protein activity, this product still generally remains safe for those with lactose intolerance.

Plant derived protein types

Plant proteins have reduced essential amino acid diversity and lack methionine, lysine and tryptophan however when combined from various sources still provide vital nourishment to individuals who abstain from animal products or for those who may have hypersensitivity allergies to animal products such as lactose intolerance or cow milk protein allergy (CMPA). The benefits of plant proteins were once based upon population based studies regarding volume of plant based food intake versus animal derived products. It's now considered that the constituents of plant based foods, such as soya protein, the most widely consumed source

of plant protein may have more than a purely nutritional role in disease prevention (Hasler, 2002)

Higher intakes of non-essential amino acids are reported among vegetarians and as such offset intakes of essential amino acids. Lower levels of lysine and methionine are reported in individuals which consume protein from plant sources and as such hyperproteinaemia is a concern (Krajcovicova-Kudlackova et al, 2005). This condition displays abnormally low blood protein concentration may become commonplace without adequate nutritional knowledge (Key *et al*, 2006). The benefits on consuming plant based proteins and reducing cardiovascular health risk is well documented within the literature (Richer et al, 2015). Plant protein hydrolysates yield abundant small molecular weight peptides that in experimental settings have displayed prevention of hypertension (Yang et al, 2004), promote heart health (Erdman, 2000) and have less total lipid and carbohydrate content which may promote weight loss, improve insulin sensitivity and promote glucose homeostasis (Tian et al, 2017). There is an overall interest for knowledge of the diet and the way in which the protein is consumed. However, dietary alternations such as abstaining from animal protein sources in favour of plant based protein sources that contain other beneficial components remain a difficult factor to control for.

Animal meat protein

Proteins obtained from animal sources (i.e Poultry, meat & fish) provide the largest selection and quantity of amino acids due to the completeness of the protein source. The overall quantity of amino acids obtained from these sources are significantly greater than the plant variants. While these protein sources contain valuable dietary protein, other constituents are present that have contrived sustenance for regularly dietary intake such as saturated fat, cholesterol and increased sodium content involved in processing.

Animal proteins act as connective tissue and structural components to support movement and activity. These proteins known as myofibrillar proteins that consist mainly of actin, myosin and titin. Stromal proteins consisting of collagen, elastin and reticulins as well as sarcoplasmic proteins containing biologically active proteins such as myoglobin (colour of muscle) and haemoglobin (oxygen transfer protein).

Protein content varies between species, with the average protein content averaging 20-25% of with remaining consisting of water, lipids and intramuscular energy stores. Controversy surround animal products extend primarily to their lipid, sodium and cancer promoting heterocyclic amine compounds when overcooked (Zheng and Lee, 2009), however as a protein source it is favoured in many situations. When comparing plant base protein to animal, animal protein favours anabolic response. In a healthy active individual the improved amino acids profile of animal protein which contain higher quantity of methonine, lysine and leucine have beneficial effects for recovery, endurance and performance (Van Vilet et al, 2015).

Novel protein sources

As the global population expands the search for sustainable macronutrient sources have proven a worthwhile endeavour. The ability to produce mass amounts of carbohydrates, lipids and protein from novel sources has also unearthed sources of minerals and macronutrients that may be exploited for health promotion. Ancestral dietary patterns are re-emerging and coined as possible ways of improving nutritional health and wellbeing over current dietary applications (Cerna, 2011). Current production of animal protein sources is seen as problematic due to mass greenhouse gas production and direct impact on environmental hygiene. Novel sourced food by-products that yields desirable macronutrients, especially protein, are of interest in a bid to reduce overall food waste and improved food utilization and as such has taken the front seat for driving research and industry to work together to identify new food sources (Batista, 1999)

Table 1.4	Seaweed (Dulse)	Insects (Crickets)	Brewers spent grain
Protein	10 – 35 %	>55 %	≥20 %
Lipids	<1 %	25 %	nd
Carbohydrates	40 – 50 %	<10 %	<1 %

Protein structure

Within the protein complex are a combination of amino acids connected in series via peptide bonds. The structure of proteins vary due to their amino acids and the amino acid sequence however rules regarding protein characteristics follow a special arrangement that define its bioactivity. Proteins contain several structural units, primary, secondary, tertiary and quaternary. For the purpose of amino acid and/or peptide liberation the primary structure of the protein contains the amino acid sequence of interest. The remaining structural characteristics define the protein shape (secondary), folding capacity (tertiary) and protein/protein interaction (quaternary).

Protein bioactivity

Nutritional approaches describe protein bioactivity as the duration it takes from protein being ingested to the amino acids being found in circulating systems. Newer approaches describe bioactivity as the affect seen on biological process directly or via substrates that have an impact on bodily systems (Jeewanthi et al, 2017). Unlike carbohydrates and lipids the bioactivity of proteins including functionality and biological activities can be improved via hydrolysis to release peptides or amino acids (Pouliot et al, 2009). The intake of protein in the general population may be for exercise, growth and repair of tissue or for recovery from illness that requires higher nutritional intake however underlying mechanisms are involved in more than a caloric role. The history of increased protein intake via supplementation is a short one however the industry continues to innovate searching for novel sources of protein and markets for these value added products. More recently hydrolysis of protein to form smaller bioactive peptides targeting exercise and endurance and furthermore health promotion has become increasingly popular. Evidence exists that di- and tri-peptides are absorbed more rapidly than free amino acid, of which a magnitude have been identified having potent bioactivity using experimental approaches (Webb, 1990; Geissler et al, 2010)

The term bioactivity is derived from foods and food components that can affect biological processes, endogenous substrates or produce subunits that have an impact on health, physical condition and bodily functions. Protein bioactivity relating to its degradation products also encompass the peptides that are liberated during digestion. Peptides from 2 to 20 amino acids in sequence are of interest due to their specific receptor affinity, agonistic or

antagonistic signalling characteristics and ability to affect biological processes (Daliri et al, 2017). Identifying the urgent need for cost effective methods for the production, screening and application of bioactive proteins that that may yield further bioactive peptides applicable to health promotion is more important now than ever before.

Hydrolysis of protein occurs when bonds between adjacent amino acids are broken. The process of hydrolysis, meaning hydro “water” and lysis “break” is the use of water molecules to uncouple and liberate amino acids or peptides from the primary protein complex. However there is a limiting caveat that only certain proteins can under hydrolysis using water. Protein structure including the tertiary portion of the protein or the folding capacity of the protein dictates its water interaction and thus the hydrolysis. As such specialised proteolytic enzymes can be used in order to overcome this hydrolysis limitation, of which will be discussed (Adler-Nissen, 1976).

Protein degradation using proteolytic enzymes

Proteolytic enzymes are capable of hydrolysing peptide bonds within a protein complex liberating peptides and amino acids. Commonly referred to as peptidases, proteases and proteinases can be found in all living organisms (Motyan et al, 2013; Barrett & McDonald, 1986). Enzymes are vitally important for physiological functioning and as such have great medical and pharmacological roles (Park & Breckenridge, 1981). The role of enzymes in biological lifecycles are now more apparent than ever with research attempting to take advantage of these enzymes and evaluate their application for industry and biotechnology. From a researching aspect, enzymes serve as imperative applications for improved life sciences from tissue dissociation, peptide synthesis, and digestion of unwanted proteins for diagnostics and therapy with the market currently worth an estimated \$3 billion in value (Sarrouh et al, 2012).

The classification of the enzymes relies on origin and target, ranging from microbial or plant derived to animal and human origin. These enzymes are further categorized based on their protein molecule interaction. The classifications can be derived due to the site of action that the enzyme interacts with the protein. Endopeptidases cleave specific peptide bonds between amino acids whereas exopeptidases target peptide bonds near the C- or N- terminus

of the peptide chain. Enzymes such as carboxypeptidases cleave single amino acids from the peptide chain whereas aminopeptidases can be used to liberate dipeptides (dipeptidyl peptidases) or tri-peptides (tripeptidyl peptidases). The application of these enzymes can be individual or used in combination or sequential hydrolysis of the intact protein. Partial hydrolysis describes when a certain molecular weight target or certain cleavage site is exhausted is achieved using 1 or more enzymes during the hydrolysis process. Complete hydrolysis described when all peptide bonds are cleaved leaving only free amino acids behind. Partial and complete hydrolysis of protein can be utilized for applications for health promotion.

The role of liberated free amino acids are well documented within the literature. It was considered that the fate of all proteins ingested orally would result in absorption via the intestinal in their individual amino acid state and while it's debated we know small peptides are able to pass through into circulation and may stimulate a biological response. Amino acids have momentous health promotion effects and regulatory roles while acting as biological catalysts affecting appetite, metabolic regulation, endocrine status, body composition, cell signalling, protein synthesis, reproduction and lactation, respectively. The process of liberating free amino acids from exogenous protein sources, while mentioned above, heavily relies endogenous enzyme activity, primarily produced within the gastric tract, mainly stomach, small intestines and pancreas following intake of food.

Summary of food derived marine peptides,

To date there has been a significant development of biologically active food proteins with clinical, therapeutic and health promoting effects beyond pure nutritional requirement. This has promoted researchers to look upon protein as a resource for potential development of protein derived molecules that may have intrinsic health promoting effects. The development of identified peptides that hold metabolic regulatory effects has expanded exponentially, however these often require injection to unlock the full potential of the molecule once a disease target has been identified.

Many of these peptide molecules that are liberated from longer protein chains have similar properties, relatively short (eg. 2 – 20 aa) and contain hydrophobic amino acids residues as

well as arginine, lysine and proline groups which are resistant to digestion when ingested orally. Smaller peptide molecules are of interest for their ability to act upon signalling mechanisms similar to larger specific molecules as well as being cheaper to manufacture. Crude bioactive peptides taken orally are of interest due to their resistance to intestinal digestion enzymes. These small molecular weight peptides are thought to pass the intestinal brush boarder and have circulatory signalling activity, as well as displaying potent post-prandial incretin effect in vivo.

Food derived peptides from gluten or casein may act as an antihypertensive with properties that can inhibit angiotensin I converting enzyme (Ibrahim et al, 2017), milk proteins containing anti-inflammatory peptides activating upon opioid receptors (Chatterton et al, 2013) and soy peptides acting upon superoxide anions and antioxidant peptides containing immunomodulatory activity (Sanchez & Vazquez, 2017). Marine proteins will however be the focus of the remainder of this chapter and further chapters. Using marine protein as a therapeutic for type 2 diabetes has been a relatively novel endeavour. The oceans are considered the richest and most untapped source of bioactive compounds (Khan et al, 2016). As population increases the need for new novel protein sources is vital and as such has led to underutilized pelagic ichthyoid species being used to generate new and novel protein and subsequent peptide hydrolysates. It is hypothesized that marine protein has the ability to yield a greater quantity of bioactive peptides that are resistant to gastrointestinal digestion, making them a potential source of nutraceutical with meaningful metabolic regulating effects.

Studies involving marine peptides have displayed anti-carcinogenic and antihypertensive abilities while further regulating circulating lipids and glucose making them of interest to metabolism and diabetes research. The typical procedure for discovery of novel bioactive peptides involves protein extraction from source, enzymatic digestion using a wide array of proteases, further fractioned using analytical machinery such as RP-HPLC or electrophoresis then assessed using bioassays and finally purified as a single bioactive peptide for further investigation. With this noted, the work presented in this chapter will contain only that of the crude protein fractions obtained via enzymatic digestion. Bioactive molecules from the crude peptide hydrolysates have been identified and as such is an area that is being actively pursued and discussed in later chapters.

The effects of the crude protein hydrolysates was first investigated using *in-vitro* bioassays. Using a concentration of protein hydrolysates (2.5 mg/ml) from various marine sources that have undergone enzymatic digestion the research was able to target diabetes specific states using tailored assays that may give a broad overview of the therapeutic potential of marine protein hydrolysates when ingested orally. In this present study, cell lines of interest were the insulin secreting BRIN-BD11, Glucagon-like-peptide 1 (GLP-1) secreting GLUTag cell line, and finally the glucose-dependant insulinotropic polypeptide (GIP) secreting STC-1 cell line. Furthermore, glucose uptake studies were performed on differentiated 3T3-L1 fibroblast cells as well as the investigation of protein/protein interaction using amylase and DPP4 enzyme bioassays while finally the toxicity of the marine protein hydrolysates were tested on each cell line mentioned using cellular proliferation and intracellular molecule production assays.

Background & Aims

There are many methods available to hydrolyze proteins into smaller peptide fractions of the parent protein molecule. The previous chapter elaborated on the hydrolysis methods used to generate numerous protein hydrolysates from novel biological marine sources using various enzyme combinations. The enzymes used ranged from single use endopeptidases, targeting specific amine groups within the protein, to sequential hydrolysis using a mixture of more than one endopeptidases and exopeptidases. The current hypothesis is that the use of a single peptidase will not yield bioactivity similar to using more than one peptidase in the generation of the protein hydrolysates. The rationale for this relies on the greater number, and variance of peptides that can be unlocked using multiple peptidases rather than a single peptidase. In order to test the hypothesis, a range of diabetes specific assays will be deployed across multiple cell lines, including the insulin secreting BRIN-BD11, GLP-1 secreting GLUTag and GIP secreting STC-1 cells. Furthermore, protein-protein interactions investigating endogenous enzyme activity in the presence of the generated protein hydrolysates such as DPP4 and Amalaysse activity will be investigated. Finally, the effect on toxicity at the cellular level using the well established LDH and MTT assays will assess any adverse effects during acute co-incubation with various hydrolysates *in vitro*. Once the bioactivity ranges across the aforementioned experiments have been assessed, the most promising hydrolysates will be carried forward to subsequent *in vivo* and human investigation.

3.2 Materials and Methods

Sample preparation of crude marine peptide hydrolysates

As discussed and detailed in chapter 2 (2.1 hydrolysate preparation), hydrolysates from several sources were screened for bioactivity *in vitro*. The hydrolysates were produced from boarfish (*Capros aper*), blue whiting (*Micromesistius poutassou*), salmon (*Salmonidae*) including separated skin and muscle trimmings and finally Dulse (*Palmaria palmata*). On extraction of intact protein subsequent hydrolysis was carried out using various proteases, outlined in section 2.2.

Acute insulin secretory activity in BRIN-BD11 cells

Insulin radioimmunoassay (RIA) was used for the determination of insulin from both insulin secreting cell lines and from ex-vivo biological plasma samples (Flatt & Bailey, 1981). Initially BRIN-BD11 cells are seeded onto 24 well plates and incubated with compounds of interest for 1 hour and subsequently collected for later analysis (described in 2.1.3) using RIA. In brief cells are seeded at a cell density of 1.5×10^5 cell/well in 1 ml of RPMI cell culture media and left for 21 hours to adhere. After 20 minutes of co-incubation with compounds of interest 900 μ l is removed from each well and stored until analysed via insulin radioimmunoassay at -20°C . A detailed outline is described in 2.2.1

GLP-1 secretion from GLUTag mouse enteroendocrine cells

On experimentation day DMEM culture media is removed, cell prepping KRBB (1 mmol/L glucose) is added for 2 hours then removed and further supplemented with KRBB of which contains 2 mmol/L glucose in all conditions (baseline) with compounds of interest used in a dose dependant manner in n=4 wells minimum. After 2 hours of co-incubation with compounds of interest 900 μ l is removed from each well and stored until analysed via ELISA at -20°C . A detailed outline is described in 2.2.2 including ELISA protocol in section 2.2.4.

GIP secretion from STC-1 mouse enteroendocrine cells

As outlined in section 2.2.3 cells were seeded on 24 well plates as described in 2.2.1. Removal of experimental supernatant were stored at -20°C until analysis. For an in-depth protocol including the GIP ELISA used see section 2.2.10.

Acute Intracellular Ca²⁺ mobilization

BRIN-BD11 cells were seeded at a cell density of 90,000 cells per well in Costar, black walled, Clear bottom 96 well plates (Appleton-Woods, Birmingham, UK) and incubated for 21 hours at 37°C (95% O₂, 5% CO₂). A fluorometric membrane potential assay was purchased from R&D Systems (Minneapolis, USA). Refer to section 2.2.11 for full experimental protocol.

Acute cellular membrane potential

A fluorescent membrane potential assay was purchased from R&D Systems (Minneapolis, USA). Using a proprietary long wavelength membrane indicator to detect the membrane potential change that is caused by the opening and closing of ion channels. The protocol was run as indicated in the manufacturer guidelines. Protocol in brief, 100 µl of working KRBB buffer supplemented with 5.6 mmol/L glucose was added to each well after removal of media. Refer to section 2.2.12 for full experimental protocol.

Cellular toxicity using LDH cytotox assay

Experimentation was carried out exactly as described in 2.1.2 with a final step of testing 50 µl of collected supernatant aliquoted into 96 well plates using test conditions (N=3) to determine LDH concentration. Using the assay kit, 50 µl of cell supernatant is mixed with 50 µl of Promega LDH substrate solution and incubated at RT protected from UV light for 30 minutes. Refer to 2.2.16 for full experimental procedure.

Cellular viability using MTT proliferation assay

Test conditions are prepared in KRBB buffer with various concentrations of glucose of which match the test conditions seen in the acute secretory activity study's, refer to section 2.1.4 for test condition preparation. For MTT experimentation protocol refer to section 2.2.15.

Statistical analysis

Data was expressed as mean \pm SEM with values compared using one-way analysis of variance (ANOVA) followed by post-hoc students T-test. Data between groups were considered statistically significant if $p \leq 0.05$. Data was plotted and analysed using Graphpad Prism 5.

3.3 Results

Insulinotropic activity of Marine protein hydrolysates

Fig 3.1 A/B - The acute insulinotropic activity during co-incubation with known insulin secretagogues in BRIN-BD11 cells.

Insulin secretion was determined over a 20 minute co-incubation with various MPH and glucose. Baseline insulin secretion was established using KRBB buffer online containing a fixed dose of glucose at 5.6 mmol/L (Fig 3.1-A) or 16.7 mmol/L (Fig 3.1-B). All other test conditions contained addition compounds from positive insulinotropic controls to a dose dependant concentration of MPH. The positive controls deployed KCL (30 mM) and GLP-1 (10^{-6}) displayed 8-fold ($p < 0.001$) and 5-fold ($p < 0.001$) insulin secretion versus baseline (5.6 mmol/L) glucose, respectively.

Fig 3.2 – 4 & 3.6 - 8 - Insulinotropic marine protein hydrolysates generated from *Palmaria palmata*

Using a fixed dose of *palmaria palmata* (ULPH018, 0.0195 – 2.5 mg/ml) in KRBB buffer containing 5.6 mmol/L glucose, the aqueous alkaline protein hydrolysate caused significant insulin secretion at 1.25 and 2.5 mg/ml MPH ($P < 0.01$ to $P < 0.001$; Fig Fig 3.2-A) respectively. This hydrolysate is used as a bioactive baseline as the peptides were generated using only aqueous hydrolysis. Subsequent hydrolysates have further undergone enzymatic hydrolysis or/and simulated gastrointestinal digestions (SGID).

The SGID variant ULPH042 had improved insulinotropic activity showing significant dose dependent insulin release from 0.312 to 2.5 mg/ml ($P < 0.05$ to $P < 0.001$; Fig 3.2 B). The enzymatically produced ULPH019 (Alcalase) similarly displayed insulin secretory activity from 0.312 to 2.5 mg/ml ($P < 0.001$; Fig 3.2 C), however after SGID, the insulinotropic activity was

lost with only 1.25 and 2.5 mg/ml showing bioactivity ($P < 0.05$ to $P < 0.01$; Fig 3.2 D). Hydrolysate ULPH020 (Alcalase/Flavourzyme) displayed cellular bioactivity and insulin secretion from 0.156 to 2.5 mg/ml of MPH ($P < 0.01$ to $P < 0.001$; Fig 3.3 A). The SGID variant, ULPH036 had lost its insulinotropic activity at 0.156 and 0.312 mg/ml, however remained potent at 0.625 to 2.5 mg/ml ($P < 0.001$; Fig 3.2 B).

The Bromelain produced hydrolysate, ULPH021 had promising insulin secretion activity displaying dose dependent bioactivity ranging from 0.078 mg/ml to 2.5 mg/ml ($P < 0.05$ to $P < 0.001$; Fig 3.3 D) however this activity was reduced after further SGID was carried out with activity seen from 0.312 to 2.5 mg/ml ($P < 0.01$ to $P < 0.001$; Fig 3.3 D), respectively. Finally the Promod produced hydrolysate, ULPH022 had insulinotropic activity from 0.625 to 2.5 mg/ml ($P < 0.01$ to $P < 0.001$; Fig 3.4 A) and had retained this activity after SGID production of ULPH040 with activity remaining from 0.625 to 2.5 mg/ml ($P < 0.05$ to $P < 0.001$; Fig 3.4 B).

The *Palmaria palmata* hydrolysates were similarly tested in supra-physiological glucose concentrations tested in KRBB supplemented with 16.7 mmol/L glucose, respectively. The positive controls deployed KCL (30 mM) and GLP-1 (10^{-6}) displayed 11 -fold and 6 -fold insulin secretion versus baseline (16.7 mmol/L) glucose (Fig 3.1 B). The aqueous produced hydrolysate, ULPH018 resulted in significant insulin secretion from 0.312 to 2.5 mg/ml ($P < 0.01$ to $P < 0.001$; Fig 3.6 A) whereas, similarly seen with the SGID variant, ULPH042 displayed improved potency and dose-dependent insulinotropic activity from 0.156 to 2.5 mg/ml ($P < 0.01$ to $P < 0.001$; Fig 3.6 B), seen also when tested at 5.6 mmol/L glucose, respectively.

The enzymatically produced hydrolysates, ULPH019 (Alcalase) resulted in significant insulin secretion from 0.625 to 2.5 mg/ml ($P < 0.001$; Fig 3.6 C), whereas some activity was lost in the SGID variant (Alcalase + SGID) where elevated insulin secretion was only reported at 1.25 to 2.5 mg/ml ($P < 0.001$; Fig 3.6 D). The Alcalase/Flavourzyme hydrolysate, ULPH020 displayed dose-dependent secretory activity from 0.312 to 2.5 mg/ml ($P < 0.01$ to $P < 0.001$; Fig 3.7 A) and retained insulinotropic potency after SGID processing, where ULPH036 resulted in activity from 0.625 to 2.5 mg/ml ($P < 0.001$; Fig 3.7 B). The Bromelain produced ULPH021 hydrolysate similarly returned insulinotropic activity from 0.625 to 2.5 mg/ml ($P < 0.01$ to $P < 0.001$; Fig 3.7 C) and further activity was observed after SGID processing and generation of ULPH038 where bioactivity was observed from 0.312 to 2.5 mg/ml ($P < 0.01$ to $P < 0.001$; Fig 3.7 D). Finally, of

the *Palmaria palmata* hydrolysates, the Promod generated ULPH022 displayed insulinotropic activity from 0.625 to 2.5 mg/ml ($P < 0.001$; Fig 3.8 A), however after further SGID processing and generation of ULPH040, dose-dependent activity was improved upon with potent insulinotropic activity displayed from 0.156 to 2.5 mg/ml ($P < 0.001$; Fig 3.8 B), respectively.

Fig 3.9 - Insulinotropic marine protein hydrolysates generated from Blue whiting

Blue whiting protein hydrolysate generated using Alcalase and Flavourzyme, ULPH063, displayed dose dependant insulinotropic activity during 20 minute co-incubation in BRIN-BD11 cells. Activity was displayed from 0.078 to 2.5 mg/ml ($P < 0.01$ to $P < 0.001$; Fig 3.9 A). The insulinotropic activity was retained after SGID processing, however only from 0.156 to 2.5 mg/ml ($P < 0.01$ to $P < 0.001$; Fig 3.9 B). Blue whiting ULPH072 protein of which only underwent SGID processing similarly displayed potent insulinotropic activity from 0.078 to 2.5 mg/ml ($P < 0.001$; Fig 3.9 C), respectively.

Fig 3.10 - Insulinotropic marine protein hydrolysates generated from Boarfish (*Capros aper*)

Boarfish hydrolysate generated using Alcalase and Flavourzyme enzymatic digestion, ULPH062, resulted in a 1.3 to 4 –fold insulin secretion versus baseline glucose control. The activity was displayed from 0.078 to 2.5 mg/ml ($P < 0.01$ to $P < 0.001$, Fig 3.10 A). Using only SGID hydrolysis, ULPH066 displayed 1.8 to 5.5 –fold insulin secretion versus basal glucose control. Activity was seen from 0.156 to 2.5 mg/ml ($P < 0.01$ to $P < 0.001$; Fig 3.10 B). Using both enzymatic digestion (Alc/Flav) and further SGID digestion, potency of hydrolysate ULPH068 was retained at higher concentrations however reduced at the previously significant lower concentrations. 1.2 to 5.8 –fold activity was displayed from 0.156 to 2.5 mg/ml ($P < 0.01$ to $P < 0.001$; Fig 3.10 C), respectively. The production of Boarfish protein hydrolysate on large scale was subsequently retested for bioactivity.

Fig 3.11 - 13 - Insulinotropic marine protein hydrolysates generated from Salmon Trimmings (*Capros aper*)

Using Alcalase, Alcalase and Flavourzyme in combination or Promod proteases with or without SGID processing, several hydrolysates were generated from Salmon muscle trimmings. The Salmon trimming hydrolysate generated using Alcalase only, ULPH051, had

displayed 2 –fold insulinotropic activity at 0.625 and 2.5 mg/ml only ($P<0.05$ and $P<0.01$; Fig 3.11 A). Further processing of ULPH051 using SGID yielded ULPH057 of which displayed improved insulin secretion of 2.5 to 2.8 –fold increase versus basal control. Insulin secretion was displayed from 0.625 to 2.5 mg/ml ($P<0.05$ to $P<0.01$; Fig 3.11 C). Furthermore, ULPH052 generated using Alcalase and Flavourzyme in combination had shown similar secretory activity ranging from 2 to 2.5 –fold higher insulin secretion versus basal glucose control. The activity was seen from 0.312 to 2.5 mg/ml ($P<0.05$ to $P<0.001$; Figure 3.11 B), however after SGID processing activity of ULPH059 was improved upon with 4 to 4.5 –fold increase in insulin secretion versus basal glucose control, respectively. Dose-dependent insulin secretion was seen from 0.625 to 2.5 mg/ml ($P<0.01$ to $P<0.001$; Fig 3.3.1-AI). Finally, hydrolysate ULPH053 generated using Promod, insulin secretion ranging from 1.8 to 3 –fold versus basal glucose control was displayed from 0.312 to 2.5 mg/ml ($P<0.05$ to $P<0.001$; Fig 3.12 A). The same hydrolysate had undergone SGID with similar activity seen ranging from 1.8 to 3.8 –fold versus basal glucose control from concentration 0.156 to 2.5 mg/ml ($P<0.05$ to $P<0.001$; Fig 3.12 B), respectively.

Incretin secretion (GLP-1) during co-incubation with marine protein hydrolysates

Fig 3.14 A - Marine protein hydrolysate incretin effect from the GLP-1 secreting enteroendocrine GLUTag cell line

The enteroendocrine GLUTag cell line was investigated for its GLP-1 secreting ability. Positive controls, Glutamine (10 mM), Forskolin (10 mM) and GIP (10^{-6}) returned from 2 to 4 –fold increased GLP-1 secretion versus 2 mM basal glucose control ($P<0.05$ to $P<0.001$; Fig 3.14 A), respectively. Using 2.5 mg/ml, all hydrolysates were subsequently co-incubated with 2 mM glucose.

Fig 3.14 - 15 - Incretin effect of marine protein hydrolysates generated from *Palmaria palmata*

During the 2 hour MPH co-incubation with 2 mM glucose, ULPH018 generated using aqueous hydrolysis only had reported 3 –fold GLP-1 secretion versus basal glucose control using 2.5

mg/ml ($P < 0.001$; Fig 3.14 B), after SGID processing, potency of ULPH042 was reduced, however elevated GLP-1 secretion was still evident ($P < 0.001$; Fig 3.14 B). Using Alcalase, ULPH019 displayed 2 fold ($P < 0.001$) GLP-1 secretion whereas its SGID variant, ULPH034 failed to increase GLP-1 secretion over the basal glucose control (Fig 3.14 C). Similarly ULPH020 promoted 2.2 fold GLP-1 secretion ($P < 0.001$) however after SGID activity was lost in ULPH036 (Fig 3.14 D). The Bromelain generated hydrolysate, ULPH021, resulted in 2 –fold ($P < 0.001$) GLP-1 secretion and retained 1.4 fold ($P < 0.05$) secretory activity after SGID generation of ULPH038 (Fig 3.15 A). Finally the Promod generated hydrolysate, ULPH022, displayed 1.6 – fold ($P < 0.001$) and SGID ULPH040, 1.4 fold ($P < 0.05$) GLP-1 secretion versus basal glucose control, (Fig 3.15 B), respectively.

Fig 3.15 C - Incretin effect of marine protein hydrolysates generated from Blue whiting

Under the same experimental conditions, blue whiting hydrolysate generated using Alcalase and Flavourzyme, ULPH063 promoted a 1.4 fold ($p < 0.01$) increase of GLP-1 secretion over basal glucose control, however after SGID processing of ULPH063 and generation of ULPH063, potency was improved upon and 1.8 –fold ($P < 0.001$) GLP-1 secretion was displayed (Fig 3.15 C).

Fig 3.15 D - Incretin effect of marine protein hydrolysates generated from Boarfish (*Capros Aper*)

Boarfish hydrolysate generated using Alcalase and Flavourzyme in combination, ULPH062 returned 0.5 -fold lower GLP-1 secretion than basal glucose control, however no effect was displayed from its SGID, ULPH068 variant. Protein hydrolysate generated using SGID only however stimulated 2.5 –fold ($P < 0.001$) increase of GLP-1 secretion over the basal glucose control (Fig 3.15 D), respectively.

Fig 3.16 - Incretin effect of marine protein hydrolysates generated from Salmon trimmings

The Alcalase generated hydrolysate, ULPH051 had no effect on cellular secretion. After further SGID processing, its ULPH057 hydrolysate caused 2.2 –fold ($P<0.001$) increase in GLP-1 secretion. The Alcalase and Flavourzyme combination, ULPH052 and its SGID variant ULPH059 both displayed potent GLP-1 secretion of 2.4 ($P<0.001$) and 2.2 –fold ($P<0.001$) increase versus basal glucose control, respectively. Hampered GLP-1 secretion was displayed from the Promod generated hydrolysate ULPH053 and its SGID ULPH053 Variant with 0.5 fold and 0.4 fold reduction in GLP-1 secretion (Fig 3.16 A).

Incretin secretion (GIP) during co-incubation with marine protein hydrolysates

Fig 3.17 A - Marine protein hydrolysate incretin effect from the GIP secreting enteroendocrine STC-1 cell line

The STC-1 cell line was investigated for its glucose-dependant insulinotropic polypeptide (GIP) secretion. Using the same experimental conditions as the GLUTag investigation, positive controls, Palmitic acid (500 μ M) and Glutamine (10 mM) reported a 4 –fold increase ($P<0.001$) of glucose-dependant insulinotropic polypeptide (GIP) versus basal glucose control respectively (Fig 3.17 A). All test conditions contained 2.5 mg/ml of various marine protein hydrolysates within a 2 mM glucose supplemented KRB buffer co-incubated for 2 hours.

Fig 3.17 B – 3.18 - GIP secretion during acute co-incubation of marine protein hydrolysates generated from *Palmaria palmata*

In the presence of ULPH018, a 4 –fold ($P<0.001$) increase of GIP secretion was displayed whereas the SGID, ULPH042 variant improved upon this stimulating a 5.8 –fold ($P<0.001$) increase in GIP secretion versus basal glucose control (Fig 3.17 B). The Alcalase generated, ULPH019 reported a 6 –fold ($P<0.001$) increase in GIP furthermore, the SGID variant, ULPH034 promoted a 8 –fold ($P<0.001$) increase in GIP secretion under the same conditions (Fig 3.17 C). The secretory activity reported from ULPH020 was mildly elevated ($P<0.01$) above basal glucose control, whereas the SGID variant reported a reduction ($P<0.01$) in GIP secretion (Fig 3.17 D). The bromelain produced ULPH 021 and Bromelain/SGID, ULPH038 increased GIP

secretion by 5 –fold ($P<0.001$) and 4 – fold ($P<0.001$), respectively (Fig 3.18 A). Finally, the Promod, ULPH022 and Promod/SGID ULPH040 improved GLP-1 secretion by 6 –fold and 3.8 –fold versus basal glucose control (Fig 3.18 B).

Fig 3.19 A - GIP secretion during acute co-incubation of marine protein hydrolysates generated from *Blue whiting*

During co-incubation with ULPH063 no GIP secretion above basal glucose control was reported, however ULPH070 and ULPH072 reported a 2 –fold ($P<0.001$) and 6 –fold ($P<0.001$) increase in GIP (Fig 3.19 A).

Fig 3.19B - GIP secretion during acute co-incubation of marine protein hydrolysates generated from *Boarfish (Capros aper)*

The boarfish hydrolysates generated using Alcalase and Flavourzyme, ULPH062 and its SGID variant, ULPH068 caused no elevation of GIP versus basal glucose control, however when hydrolysed with only SGID, ULPH066 promoted a 5 –fold increase above basal levels($P<0.001$) of GIP (Fig 3.19 B).

Fig 3.19 C - D - Incretin effect of marine protein hydrolysates generated from Salmon trimmings

Salmon trimming hydrolysate ULPH generated using SGID promoted a 3.8 –fold increase ($P<0.001$) in GIP secretion versus basal glucose control (Fig 3.19 C). Salmon skin gelatine, ULPH032 caused a 2 –fold increase in GIP secretion, whereas ULPH 24/26/28 & 30 caused no significant elevation of GIP above basal glucose control, respectively (Fig 3.19 D).

Establishing glucose uptake in transdifferentiated adipocytes during co-incubation with marine protein hydrolysates

Fig 3.20 A - Glucose uptake investigation during acute co-incubation using marine protein hydrolysates on differentiated 3T3-L1 fibroblast cells

The 3T3-L1 cell line was investigated after it had undergone trans-differentiation from fibroblast to adipocyte cells. This was done over a period of 9 days, for more information please refer to section 2.2.3. The positive control apegenin results in significant reduction ($P < 0.001$) of glucose uptake via the inhibition of the GLUT1 receptor whereas insulin (1 nM & 100 nM) had the opposite effect causing 1.4 ($P < 0.001$) and 1.8 ($P < 0.001$) fold glucose uptake versus the basal glucose control (Fig 3.20 a). The remainder of the test conditions remained consistent with marine hydrolysates used at 2.5 mg/ml on their own or in the presence of insulin (1 nM) for the duration of the experiment.

Fig 3.20 B – 3.21 B - Glucose uptake assessment during acute co-incubation using *Palmaria palmata* hydrolysate

ULPH018 caused no significant uptake of glucose on its own, however in the presence of insulin had displayed a 1.5 –fold increase ($P < 0.05$). The SGID variant ULPH042 caused a significant uptake of glucose on its own, however this effect was lost in the presence of insulin (Fig 3.20 B). When co-incubated, ULPH019 and ULPH042 both caused a similar 1.5 –fold ($P < 0.001$) increase in glucose uptake, however the same result was shown when combined with insulin with no additive effect displayed ($P < 0.001$; Fig 3.20 C). Additionally ULPH020 caused a 1.8 fold increase ($P < 0.001$) in glucose uptake whereas in combination with insulin results in a 1.7 fold increase ($P < 0.001$) versus basal glucose control (Fig 3.20 D). Similar results were displayed with ULPH036 with and without insulin present, with a 1.38 –fold ($P < 0.05$) seen from both test conditions . The ULPH021 failed to improve glucose uptake by when incubated alone, with improvement in glucose uptake seen when incubated with insulin with a 1.5 –fold increase ($P < 0.01$; Fig 3.21 A). The ULPH038 SGID variant resulted in no improvement in glucose uptake with and without insulin. (Fig 3.21 A). Finally ULPH022 when in the presence of insulin improved glucose uptake by 1.5 –fold increase ($P < 0.05$) while the SGID ULPH040 having no effect in the presence of insulin or on its own (Fig 3.22 B).

Fig 3.21 C - Glucose uptake assessment during acute co-incubation using *Boarfish* hydrolysate

Glucose uptake was promoted with ULPH062 ($P < 0.05$) however in the presence of insulin the uptake was diminished. Improvement in uptake was seen with ULPH068 with ($P < 0.01$) and without ($P < 0.05$) insulin causing similarly significant 1.3 –to 1.4 –fold increase in glucose uptake (Fig 3.3.4G). Finally both ULPH066, with ($P < 0.001$) and without ($P < 0.01$) insulin promoted a 1.4 to 1.5 –fold increase of glucose uptake (Fig 3.21 C), respectively.

Fig 3.21 D - Glucose uptake assessment during acute co-incubation with *Blue whiting* hydrolysate

Glucose uptake using ULPH063 and ULPH072 was promoted with ($P < 0.01$) and without insulin ($P < 0.05$ to $P < 0.01$). In the presence of both hydrolysates, uptake was increase on average 1.4 to 1.5 fold during co-incubation, However ULPH070 failed to promote glucose uptake in adipocyte cells under any test condition, (Fig 3.21 D), respectively.

Establishing amylase activity during co-incubation with marine protein hydrolysates

Fig 3.22 A - Inhibition of start digestion using various marine protein hydrolysates

Starch, a long chain polysaccharide, digested in the presence of amylase (2 U/ml). In the presence of the positive control, Acarbose, of which inhibits amylase activity, starch digestion was reduced by over 95% ($p < 0.001$). Further test conditions used 2.5 mg/ml of hydrolysate of interest in the presence of amylase (2 U/ml) and starch (2 mg/ml) (Fig 3.22 A).

Fig 3.22 B - Inhibition of amylase using *Palmaria palmata* protein hydrolysates

Hydrolysates generated from palmaria palmata displayed a similar reduction in start digestion across ULPH 042 (-18%) 019 (-20%) 034 (-21%) 020 (-21%) 036 (-19%) 021 (-21%) 038 (-15%)

022 (-15%) 040 (-18%) ($P < 0.001$) with the ULPH018 generated using aqueous hydrolysis resulting in 28% reduction ($P < 0.001$) of amylase activity (Fig 3.22 B), respectively.

Fig 3.22 C - Inhibition of amylase using Blue whiting protein hydrolysates

Hydrolysate ULPH 063 and ULPH 072 had no effect on reducing amylase activity whereas ULPH070 reduce amylase activity by 18% ($p < 0.01$; Fig 3.22 C).

Fig 3.22 D - Inhibition of amylase during co-incubation with Boarfish hydrolysates

Comparative to the amylase online control, none of the boarfish hydrolysates investigated reduced amylase activity (Fig 3.22 D)

Fig 3.23 - Inhibition of amylase during co-incubation with Salmon trimmings

Comparative to the amylase online control, none of the salmon trimmings hydrolysates investigated reduced amylase activity (Fig 3.23 A)

Establishing DPP-4 activity during co-incubation with marine protein hydrolysates

Fig 3.24 A - Inhibition of Dipeptidyl peptidase IV (DPP4) using various marine protein hydrolysates

Marine protein hydrolysates were investigated for their ability to interact and potential inhibit the enzymatic activity of the serine protease, DPP4. The positive control, Diprotin A (DPA, Ile-Pro-Ile) was used due to the interaction and reduction of activity between the molecule and DPP4. A dose dependant reduction of DPP4 activity was displayed from 2.5 to 200 μ M of DPA ranging from 10% to 95% reduction ($P < 0.01$ to $P < 0.001$) in DPP4 activity (Fig 3.24 A). Similar

experimental controls were used, and all other marine hydrolysates were investigated using 2.5 mg/ml in the presence of 8 mU/ml DPP4 and the DPP4 luminescent substrate Gly-Pro-AMC (200 μ M).

Fig 3.24 B – 3.25 B - Inhibition of Dipeptidyl peptidase IV (DPP4) using *Palmaria palmata* protein hydrolysates

The DPP4 activity was reduced by 32% ($P < 0.001$) during co-incubation with ULPH018 and similarly its SGID variant, ULPH042, displayed a 30% ($P < 0.05$) reduction in enzyme activity (Fig 3.24 B). Similarly, ULPH019 and reduced enzyme activity by 34% ($P < 0.001$) and to a lesser extent, ULPH034 reduced activity by 17% ($P < 0.001$; Fig 3.24 C). Enzyme activity during ULPH020 co-incubation was reduced by 35% ($P < 0.001$) whereas its SGID variant displayed a 17% reduction ($P < 0.05$; Fig 3.24 D). While ULPH021 had shown a 10% reduction ($P < 0.01$) in enzyme activity, the SGID variant improved upon DPP4 enzyme inhibition with activity reduced by 20% ($P < 0.001$; Fig 3.25 A). Finally ULPH022 displayed a 14% reduction ($P < 0.001$) while ULPH040 inhibited DPP4 by 19% ($P < 0.001$; Fig 3.25 B), respectively.

Fig 3.25 C - Inhibition of Dipeptidyl peptidase IV (DPP4) using *Boarfish* protein hydrolysates

Boarfish protein hydrolysate, ULPH062 reduced DPP4 enzyme activity by 15% ($P < 0.01$) reduction in DPP4 activity (Fig 3.25 D).

Fig 3.25 D - Inhibition of Dipeptidyl peptidase IV (DPP4) using Blue whiting protein hydrolysates

Blue whiting hydrolysate, ULPH063 displayed a 15% reduction ($P < 0.05$) in DPP4 enzyme activity (Fig 3.25 C).

Fig 3.26 A / B - Inhibition of Dipeptidyl peptidase IV (DPP4) using *Salmon* hydrolysates

Salmon gelatine hydrolysate, ULPH026 and Salmon trimmings, ULPH052 and displayed a 22% and 29% total reduction ($P < 0.001$) in DPP4 enzyme activity (Fig 3.26 A/B).

Measurement of cAMP during co-incubation with marine protein hydrolysates

Fig 3.27 A - Measurement of intracellular cAMP production during co-incubation with various MPH

Using BRIN-BD11 cells, the signalling mechanism of action was assessed via the production of intracellular cAMP. Using positive controls, elevated intracellular cAMP was reported using 16.7 mmol/L glucose ($P<0.05$) and GLP-1 ($P<0.001$) comparative to the glucose only basal control of 5.6 mmol/L glucose only (Fig 3.27 A). The remaining test conditions used a fixed dose of 2.5 mg/ml of marine protein hydrolysates in KRBB supplemented with 5.6 mmol/L glucose. Results are comparative to basal glucose control, respectively.

Fig 3.27 B - Measurement of intracellular cAMP production during co-incubation with Blue whiting MPH

Across the blue whiting hydrolysates investigate, ULPH63, ULPH070 and ULPH072 increased intracellular cAMP production similarly by 60% ($P<0.001$; Fig 3.27 B) versus the basal glucose control, respectively.

Fig 3.27 C - Measurement of intracellular cAMP production during co-incubation with Boarfish MPH

Investigating ULPH066, ULPH062 and ULPH068 increased ($P<0.001$) cAMP production similarly by 50% above basal glucose control (Fig 3.27 C).

Fig 3.27 D - Measurement of intracellular cAMP production during co-incubation with Salmon gelatine MPH

The Salmon gelatine generated ULPH026 failed to stimulate cAMP production during experimentation, however ULPH026, 030 and 053 increased cAMP production by approximately 80% ($P<0.001$) across each hydrolysate versus basal glucose control (Fig 3.27 D).

Fig 3.28 A - Measurement of intracellular cAMP production during co-incubation with Salmon trimming MPH

Salmon trimming hydrolysate, ULPH051 while displaying an upward trend of cAMP production failed to display a significant increase. The remaining hydrolysates, ULPH052, 053, 055, 057, 059, 061 and 064 significantly increased intracellular cAMP production above basal glucose control (P<0.05 to P<0.001; Fig 3.28 A).

Fig 3.28 B - Measurement of intracellular cAMP production during co-incubation with *Palmaria palmata* MPH

Elevated cAMP production was evident in several of the *Palmaria palmata* hydrolysates. There was approximately 25% increase (P<0.05 to P<0.001) in cAMP production across all hydrolysates, except ULPH018 and ULPH040 where no significant elevation of cAMP was recorded. Hydrolysate ULPH020 and its SGID variant ULPH036 increased intracellular cAMP production by 80% (P<0.001) versus the basal glucose control (Fig 3.28 B).

Measurement of intracellular Ca²⁺ during co-incubation with marine protein hydrolysates

Fig 3.29 A - Measurement of intracellular calcium mobilization during co-incubation with various MPH

Using a fluorescent-based assay for detecting changes in intracellular calcium was deployed to establish further mechanisms involved in cellular signalling. Positive controls of which effect calcium mobilization were deployed such as alanine (10 mM). During co-incubation Alanine acts a calcium-specific agent that is essential to membrane binding. During experimentation, Alanine caused a 15 –fold increase (P<0.001) in calcium mobilization versus glucose only control (Fig 3.29 A), respectively.

Fig 3.29 B - Measurement of intracellular calcium mobilization during co-incubation with *Palmaria palmata* MPH

During investigation, *Palmaria palmata* generated with Alcalase and Flavourzyme in combination calcium mobilization was increased 80 –fold (P<0.001; Fig 3.29 B) versus the glucose only control.

Fig 3.30 A - Measurement of intracellular calcium mobilization during co-incubation with Salmon trimmings MPH

During investigation, Salmon trimmings generated with Alcalase and Flavourzyme in combination calcium mobilization was increased 15 –fold ($P<0.001$; Fig 3.30 A) versus the glucose only control.

Fig 3.30 B - Measurement of intracellular calcium mobilization during co-incubation with Boarfish MPH

During investigation, Boarfish generated with Alcalase and Flavourzyme in combination calcium mobilization was increased 22 –fold ($P<0.001$; Fig 3.30 B) versus the glucose only control.

Fig 3.31 A - Measurement of intracellular calcium mobilization during co-incubation with Blue whiting MPH

During investigation, Blue whiting generated with Alcalase and Flavourzyme in combination calcium mobilization was increased 40 –fold ($P<0.001$; Fig 3.31 A) versus the glucose only control.

Measurement of membrane potential during co-incubation with marine protein hydrolysates

Fig 3.32 A - Membrane potential activation during co-incubation with various MPH

Signalling effects that cause membrane potential are carried out via ion channel sensitivity and activation. The positive control for investigation of BRIN-BD11 insulin secreting cells was potassium chloride (KCL) a potent membrane potentiating insulinotropic electrolyte. Under experimental conditions, KCL caused a 75 –fold peak increase ($P<0.001$) in membrane potential during acute co-incubation (Fig 3.32 A).

Fig 3.32 A - Acute membrane potential activity during co-incubation with Salmon skin gelatine MPH

During acute co-incubation with salmon skin gelatine returned a 50 –fold peak increase ($P<0.001$) in membrane potential versus glucose only control in BRIN-BD11 cells (Fig 3.32 A).

Fig 3.32 B - Acute membrane potential activity during co-incubation with *Palmaria palmata* MPH

During acute co-incubation with *Palmaria palmata* returned a 125 –fold peak increase ($P<0.001$) in membrane potential versus glucose only control in BRIN-BD11 cells (Fig 3.32 B).

Fig 3.33 A - Acute membrane potential activity during co-incubation with *Salmon trimmings* MPH

During acute co-incubation with *salmon trimmings* returned a 55 –fold peak increase ($P<0.001$) in membrane potential versus glucose only control in BRIN-BD11 cells (Fig 3.33 A).

Fig 3.33 B - Acute membrane potential activity during co-incubation with *Boarfish* MPH

During acute co-incubation with *Boarfish* returned a 25 –fold peak increase ($P<0.001$) in membrane potential versus glucose only control in BRIN-BD11 cells (Fig 3.33 B).

Fig 3.34 A - Acute membrane potential activity during co-incubation with *blue whiting* MPH

During acute co-incubation with blue whiting returned a 25 –fold peak increase ($P<0.001$) in membrane potential versus glucose only control in BRIN-BD11 cells (Fig 3.34 A).

Measurement of cellular toxicity during co-incubation with marine protein hydrolysates

Fig 3.35 A - Investigation of LDH release and cellular toxicity during acute co-incubation with various MPHs

Intracellular LDH release was investigated using the Promega cytotox-96 non-radioactive LDH assay. Using various positive controls the supernatant was used to test LDH concentration versus a basal glucose control. During investigation, none of the positive

controls used displayed elevated LDH release versus the basal control (Fig 3.35 A). The target marine protein hydrolysate for LDH release used was 2.5 mg/ml and comparative to the basal glucose control, respectively.

Fig 3.36 A - Cellular LDH release during co-incubation with *Palmaria palmata* MPH

During investigation, all hydrolysates derived from *Palmaria palmata* caused apparent significant cellular LDH release ($P < 0.001$; Fig 3.36 A) suggesting cellular toxicity, however this will be further investigated using MTT cellular respiration assay.

Fig 3.36 B - Cellular LDH release during co-incubation with *Boarfish*, *blue whiting*, *salmon gelatine* and *salmon trimmings* MPH

During co-incubation, marine protein hydrolysates generated from boarfish, blue whiting, salmon gelatine or salmon trimmings caused no increased LDH release versus the basal glucose control, respectively (Fig 3.36 B).

Measurement of cellular respiration and proliferation during co-incubation with marine protein hydrolysates

Fig 3.37 A - Cellular viability and function investigation using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay at 5.6 mmol/L glucose

Using the redox potential in actively respiring cells to investigate cell functionality was deployed as a further measure to test *Palmaria palmata* MPH toxicity seen during the LDH release assay. Positive controls used were 20 mmol/L glucose of which acutely increased cellular viability ($P < 0.05$) whereas potent cellular toxicity was seen during co-incubation with 1 mmol/L hydrogen peroxide (H_2O_2) and a 90% loss in cellular viability ($P < 0.001$; Fig 3.37 A).

Fig 3.37 B Cellular viability and function investigation using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay at 16.7 mmol/L glucose

During investigation, none of the *Palmaria palmata* hydrolysates caused any decrease in cellular viability versus the glucose only basal control at 5.6 mmol/L (Fig 3.3.11-A) or at 16.7 mmol/L glucose (Fig 3.37 B).

Fig 3.38 A - Cellular viability during co-incubation of *Palmaria palmata* MPH in GLUTag cells

During investigation, none of the *Palmaria palmata* hydrolysates caused any decrease in cellular viability versus the glucose only basal control at 2 mmol/L (Fig 3.38 A).

Fig 3.38 B - Cellular viability during co-incubation of *Palmaria palmata* MPH in STC-1 cells

During investigation, none of the *Palmaria palmata* hydrolysates caused any decrease in cellular viability versus the glucose only basal control at 2 mmol/L (Fig 3.38 B).

Fig 3.1

A

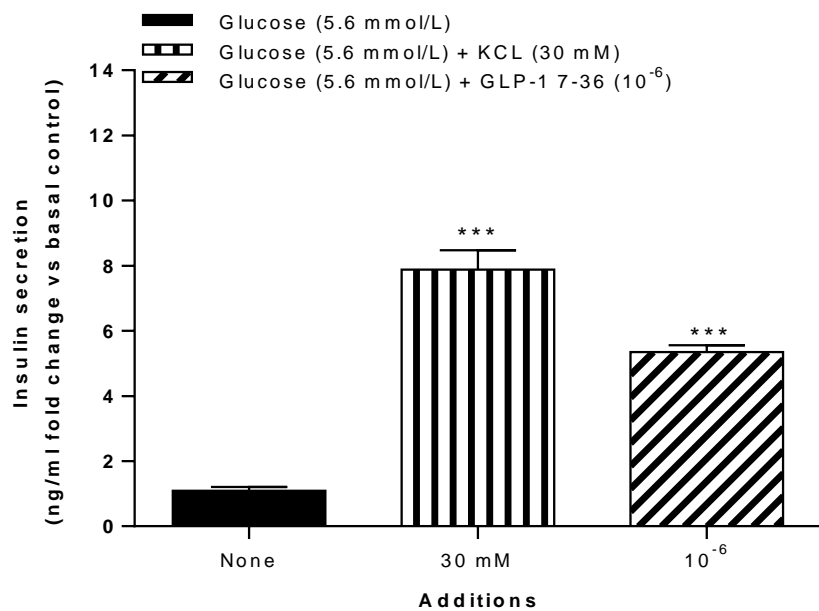


Fig 3.1.A Effects of KCL (30 mM) and GLP-1 (10⁻⁶) on insulin secretion from BRIN-BD11 cells at 5.6 mM glucose. BRIN-BD11 cells were incubated test conditions for 20 min and insulin release measured by RIA. Values are expressed as mean ± S.E.M. (n=8). ***p<0.001 compared to respective glucose control.

B

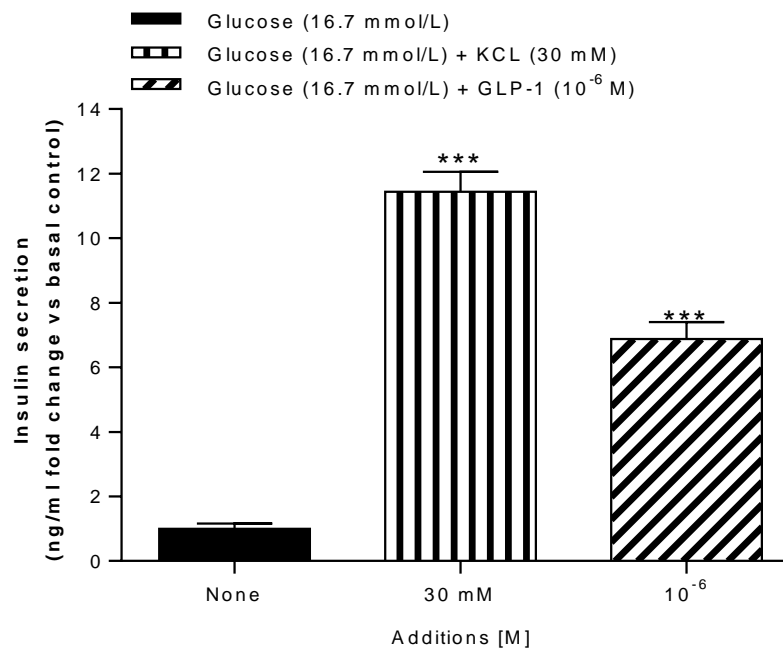


Fig 3.1.B. Effects of KCL (30 mM) and GLP-1 (10⁻⁶) on insulin secretion from BRIN-BD11 cells at 16.7 mM glucose. BRIN-BD11 cells were incubated test conditions for 20 min and insulin release measured by RIA. Values are expressed as mean ± S.E.M. (n=8). ***p<0.001 compared to respective glucose control.

Fig 3.2

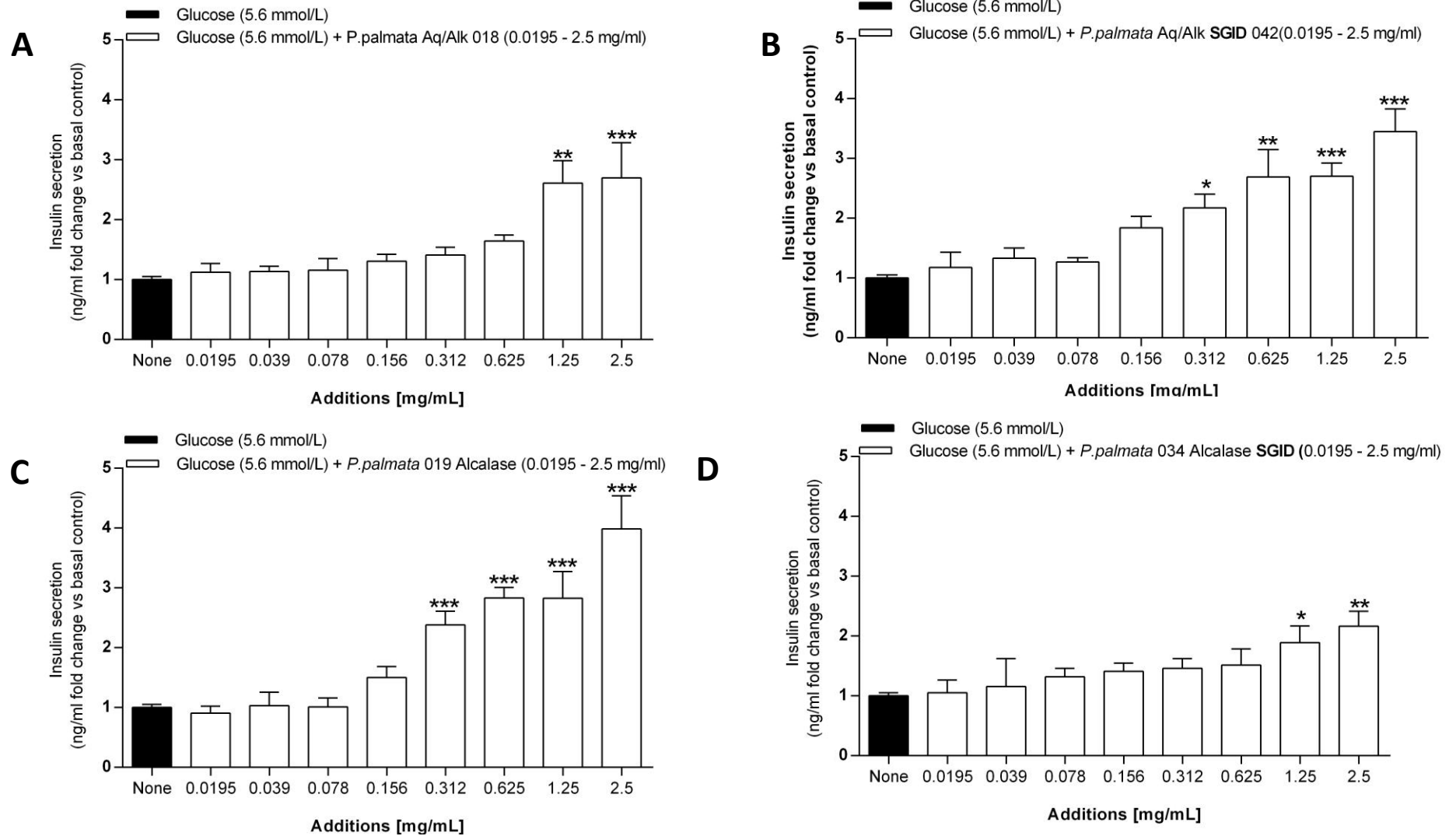


Fig 3.2.C/F. Dose-dependent effects of *Palmaria palmata* on insulin secretion from BRIN-BD11 cells at 5.6 mM glucose. BRIN-BD11 cells were incubated with either ULPH018 (Panel C), ULPH042 (Panel D), ULPH019 (Panel E) or ULPH034 (Panel F) using a range of concentrations (2.5 to 0.039 mg/ml) for 20 min and insulin release measured by RIA. Values are expressed as mean \pm S.E.M. (n=8). *p<0.05, **p<0.01, ***p<0.001 compared to respective glucose control.

Fig 3.3

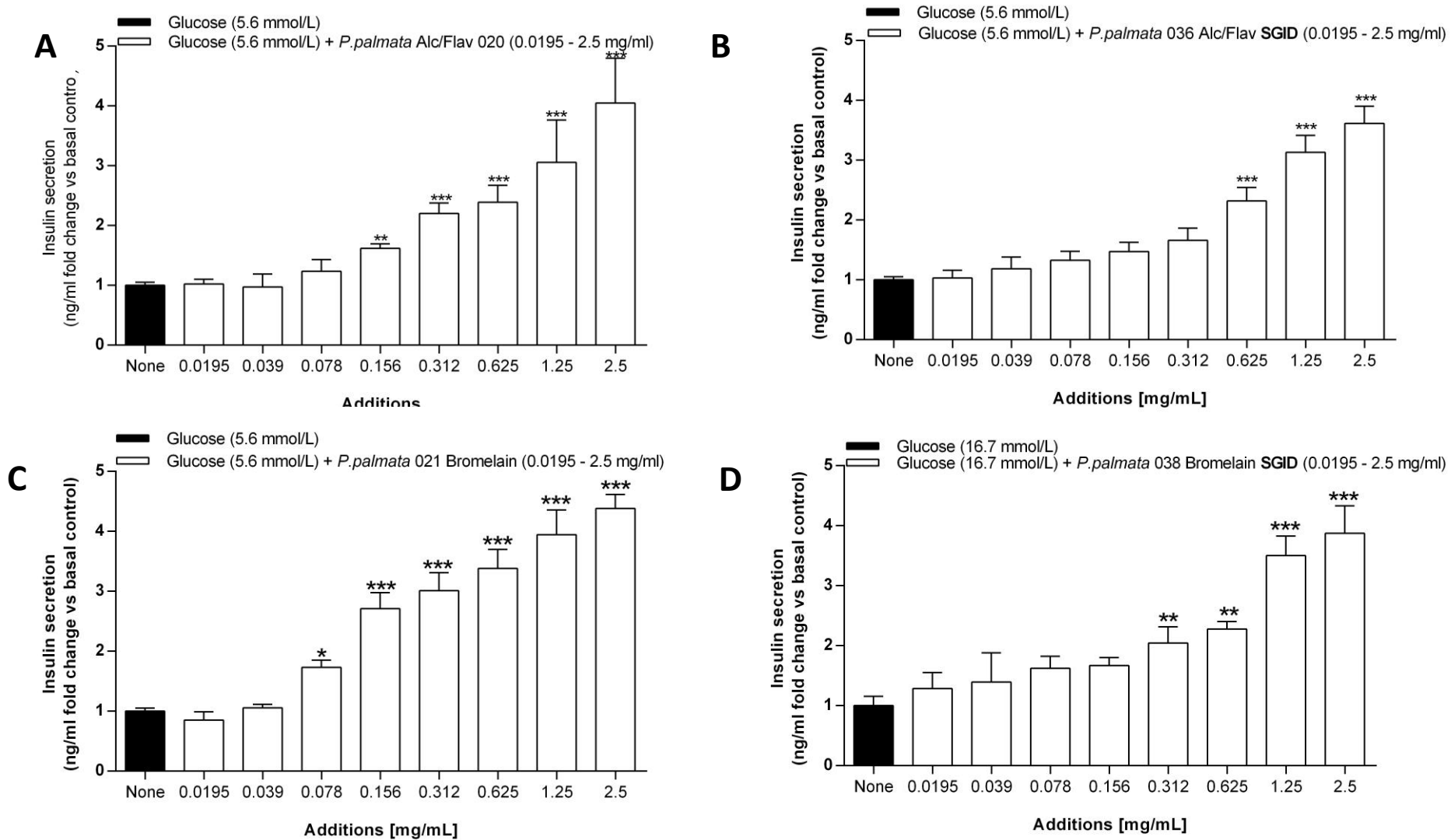


Fig 3.3. Dose-dependent effects of *Palmaria palmata* on insulin secretion from BRIN-BD11 cells at 5.6 mM glucose. BRIN-BD11 cells were incubated with either ULPH020 (Panel A), ULPH036 (Panel B), ULPH0021 (Panel C) or ULPH038 (Panel D) using a range of concentrations (2.5 to 0.039 mg/ml) for 20 min and insulin release measured by RIA. Values are expressed as mean \pm S.E.M. (n=8). *p<0.05, **p<0.01, ***p<0.001 compared to respective glucose control.

Fig 3.4

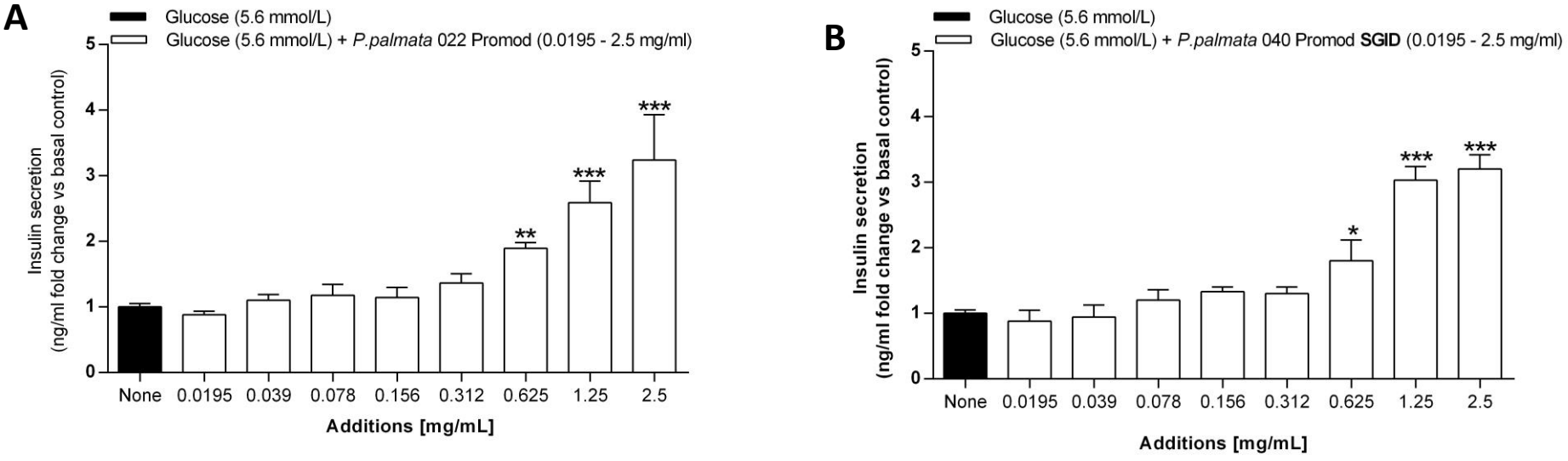
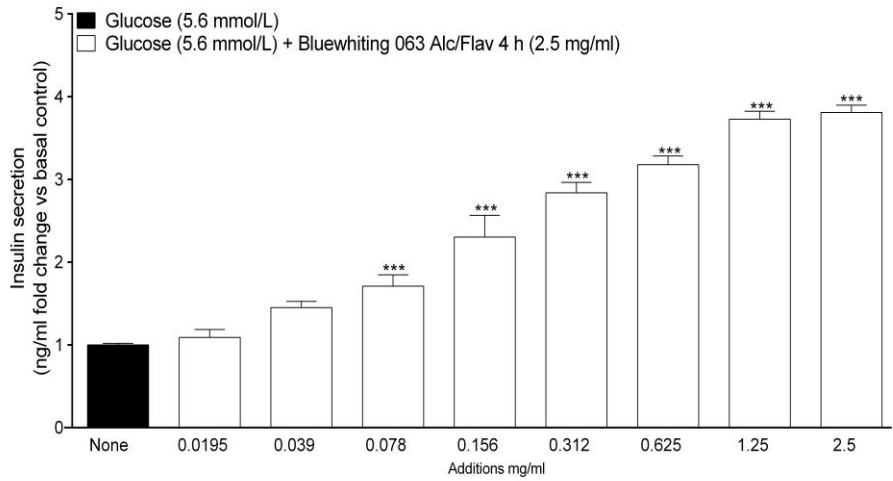


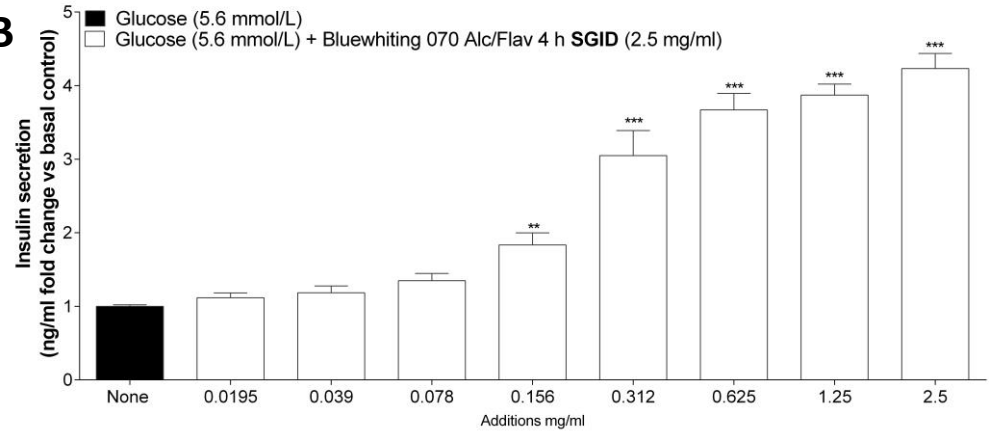
Fig 3.4. Dose-dependent effects of *Palmaria palmata* on insulin secretion from BRIN-BD11 cells at 5.6 mM glucose. BRIN-BD11 cells were incubated with either ULPH022 (**Panel A**) or ULPH040 (**Panel B**) using a range of concentrations (2.5 to 0.039 mg/ml) for 20 min and insulin release measured by RIA. Values are expressed as mean \pm S.E.M. (n=8). *p<0.05, **p<0.01, ***p<0.001 compared to respective glucose control.

Fig 3.5

A



B



C

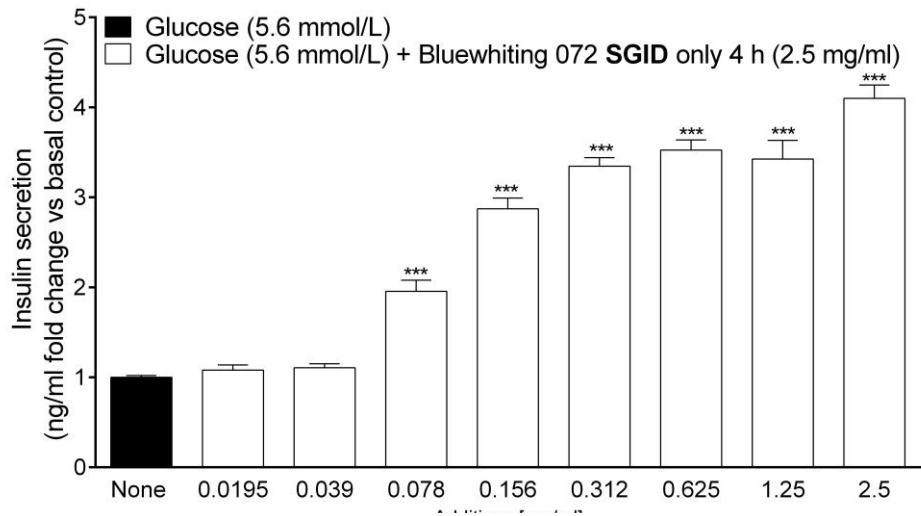


Fig 3.5 Dose-dependent effects of Blue whiting on insulin secretion from BRIN-BD11 cells at 5.6 mM glucose. BRIN-BD11 cells were incubated with either ULPH063 (**Panel A**), ULPH070 (**Panel B**) or ULPH072 (**Panel C**) using a range of concentrations (2.5 to 0.039 mg/ml) for 20 min and insulin release measured by RIA. Values are expressed as mean \pm S.E.M. (n=8). *p<0.05, **p<0.01, ***p<0.001 compared to respective glucose control.

Fig 3.6

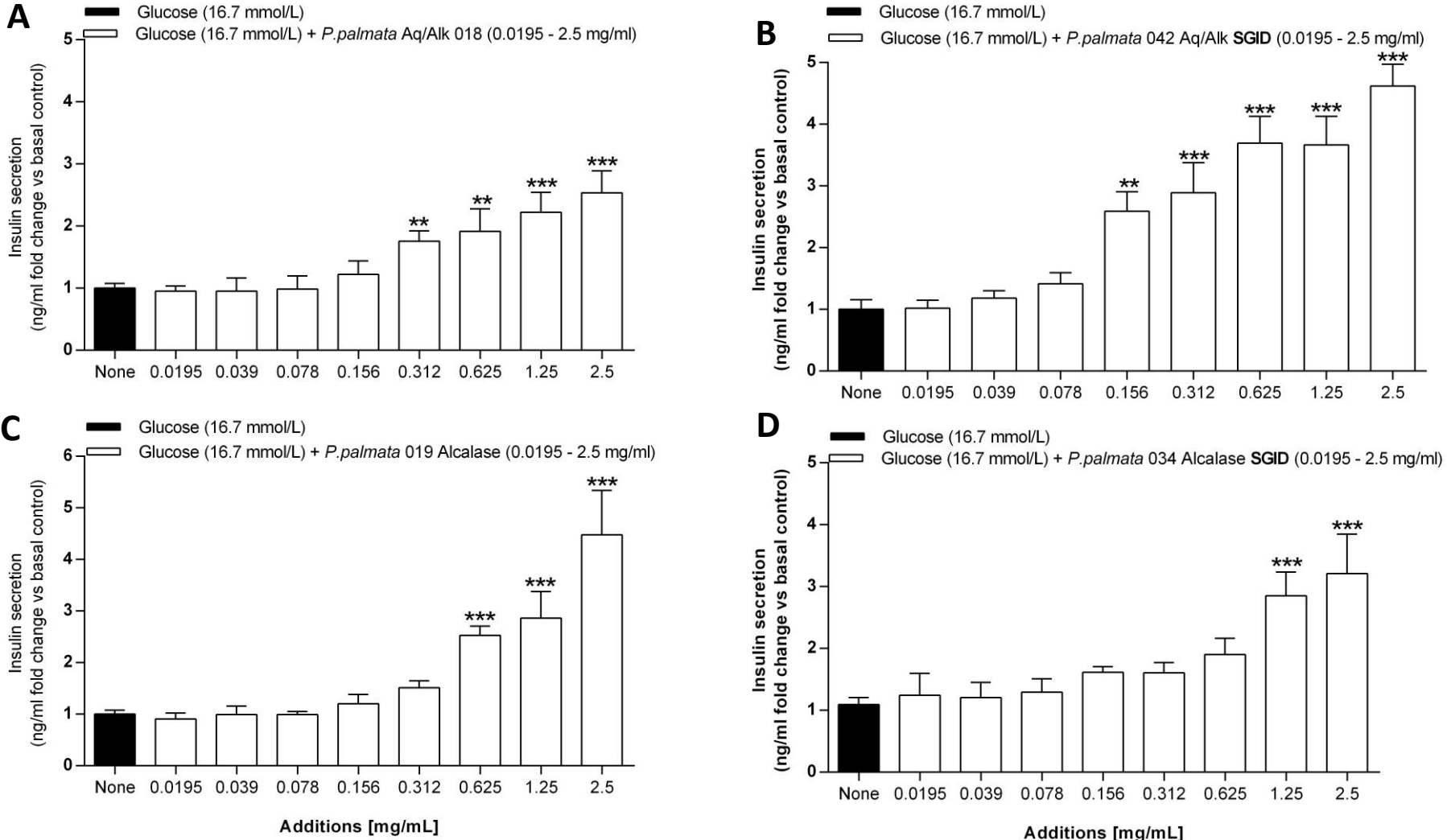


Fig 3.6. Dose-dependent effects of *Palmaria palmata* on insulin secretion from BRIN-BD11 cells at 16.7 mM glucose. BRIN-BD11 cells were incubated with either ULPH018 (Panel A), ULPH042 (Panel B), ULPH019 (Panel C) or ULPH034 (Panel D) using a range of concentrations (2.5 to 0.039 mg/ml) for 20 min and insulin release measured by RIA. Values are expressed as mean \pm S.E.M. (n=8). *p<0.05, **p<0.01, ***p<0.001 compared to respective glucose control.

Fig 3.7

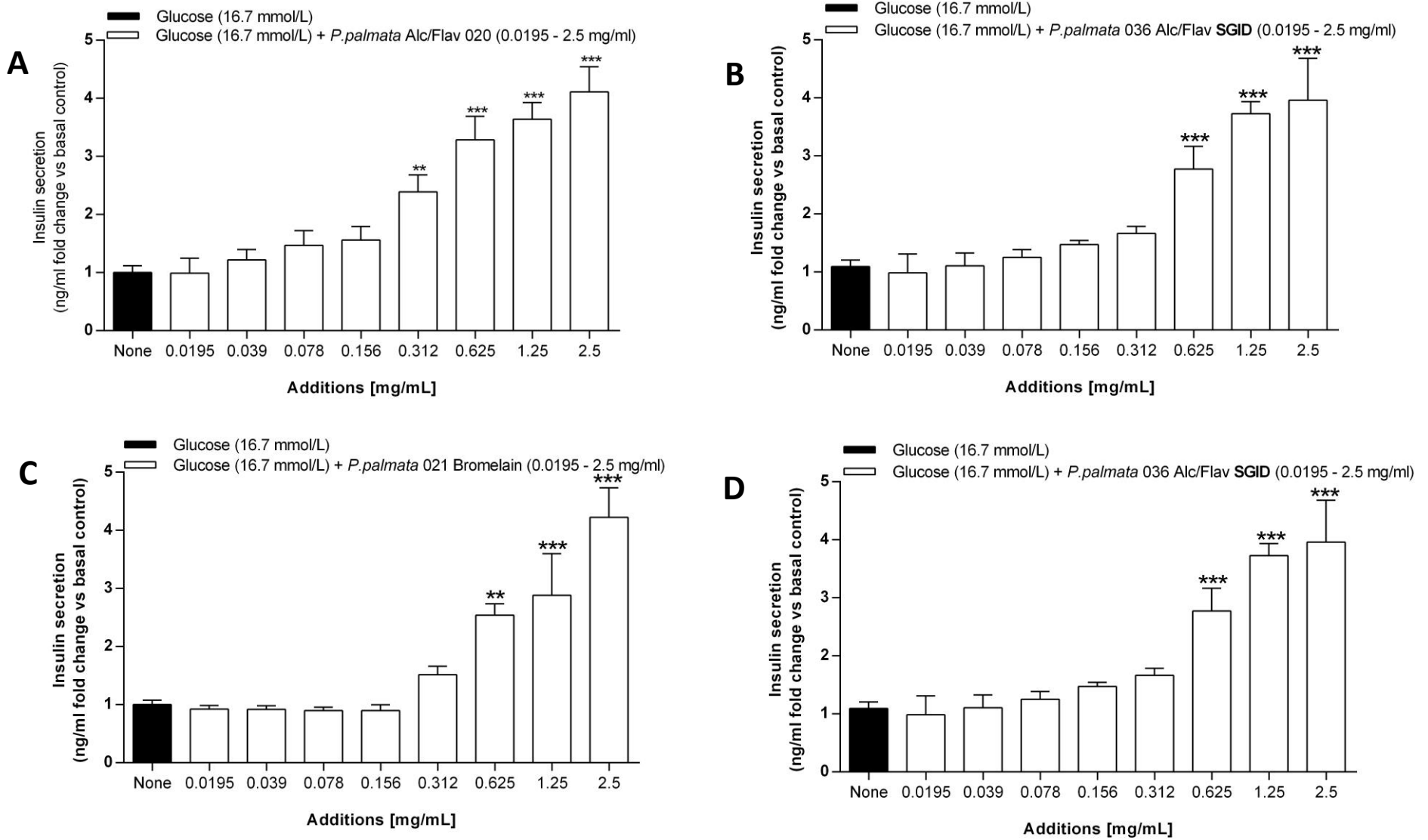


Fig 3.7. Dose-dependent effects of *Palmaria palmata* on insulin secretion from BRIN-BD11 cells at 16.7 mM glucose. BRIN-BD11 cells were incubated with either ULPH020 (Panel A), ULPH036 (Panel B), ULPH0021 (Panel C) or ULPH038 (Panel D) using a range of concentrations (2.5 to 0.039 mg/ml) for 20 min and insulin release measured by RIA. Values are expressed as mean \pm S.E.M. (n=8). *p<0.05, **p<0.01, ***p<0.001 compared to respective glucose control.

Fig 3.8

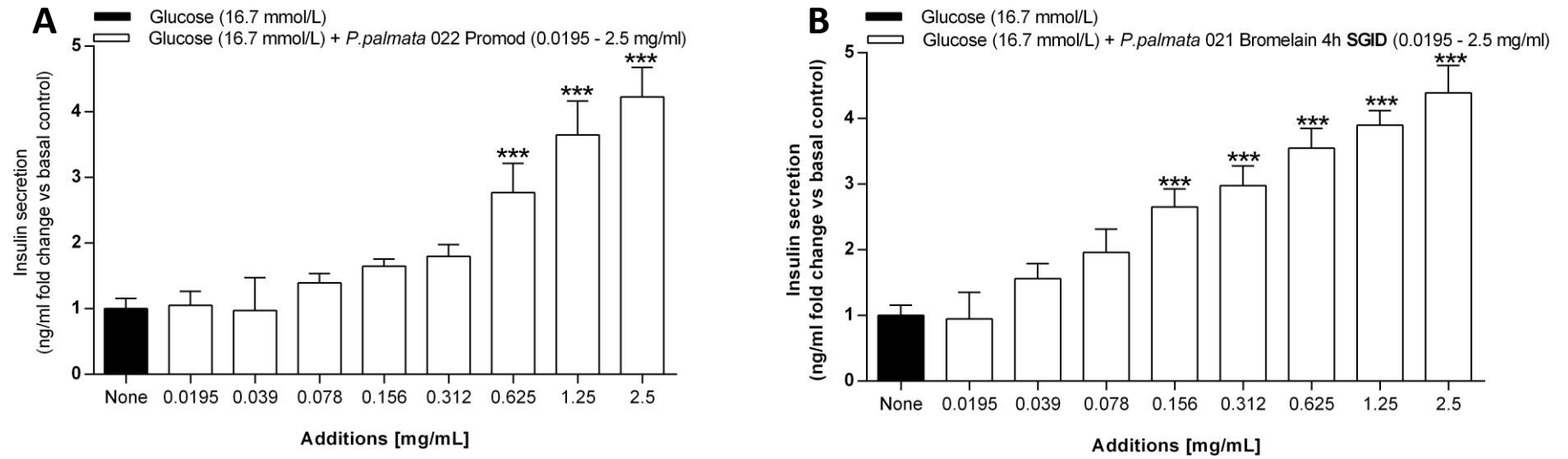


Fig 3.8. Dose-dependent effects of *Palmaria palmata* on insulin secretion from BRIN-BD11 cells at 16.7 mM glucose. BRIN-BD11 cells were incubated with either ULPH022 (**Panel A**) or ULPH040 (**Panel B**) using a range of concentrations (2.5 to 0.039 mg/ml) for 20 min and insulin release measured by RIA. Values are expressed as mean \pm S.E.M. (n=8). *p<0.05, **p<0.01, ***p<0.001 compared to respective glucose control.

Fig 3.9

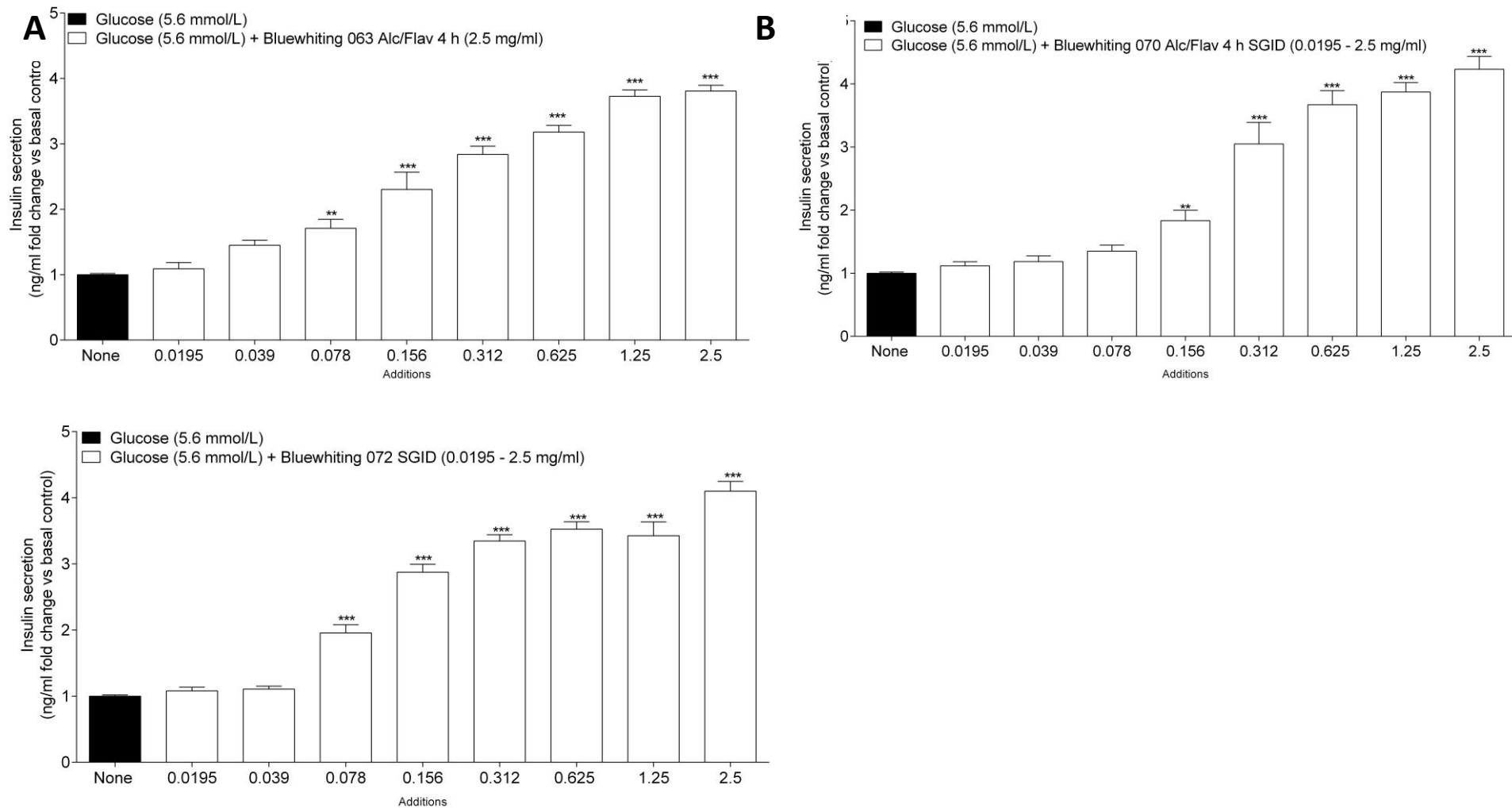
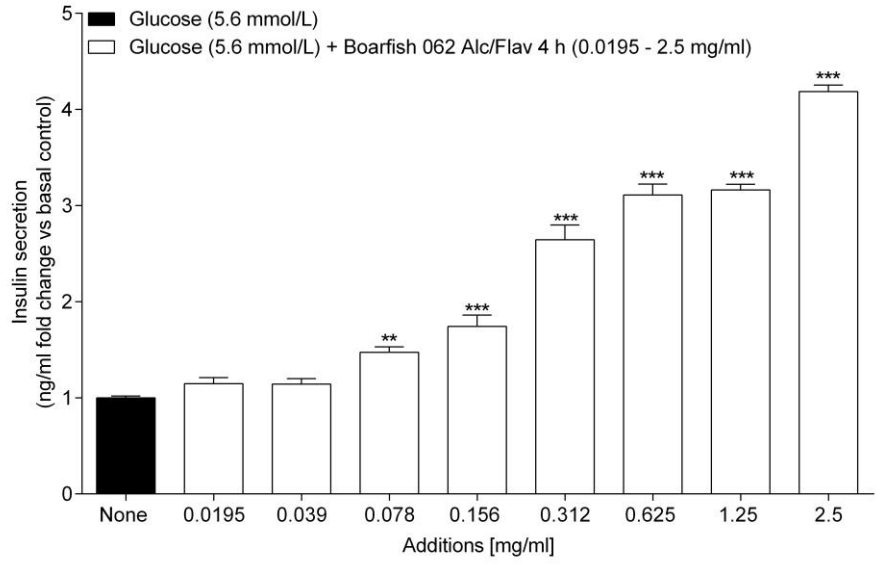


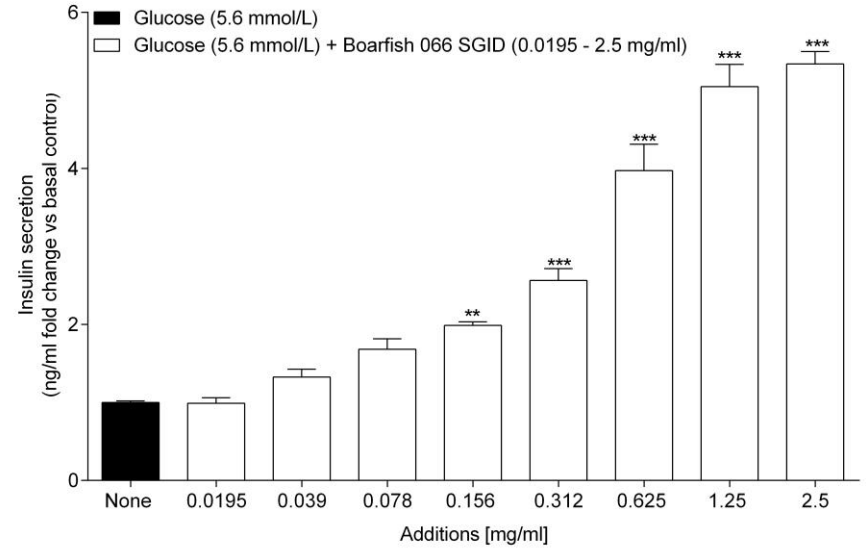
Fig 3.9. Dose-dependent effects of Blue whiting on insulin secretion from BRIN-BD11 cells at 16.7 mM glucose. BRIN-BD11 cells were incubated with either ULPH063 (**Panel A**), ULPH070 (**Panel B**), or ULPH072 (**Panel C**) using a range of concentrations (2.5 to 0.0195 mg/ml) for 20 min and insulin release measured by RIA. Values are expressed as mean \pm S.E.M. (n=8). **p<0.01, ***p<0.001 compared to respective glucose control.

Fig 3.10

A



B



C

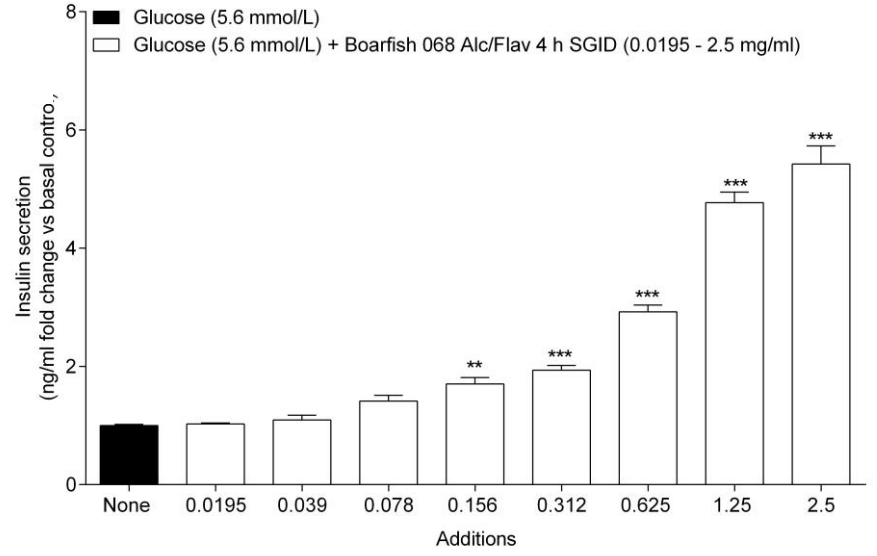


Fig 3.10. Dose-dependent effects of Boarfish on insulin secretion from BRIN-BD11 cells at 16.7 mM glucose. BRIN-BD11 cells were incubated with either ULPH062 (Panel A), ULPH066 (Panel B), or ULPH068 (Panel C) using a range of concentrations (2.5 to 0.0195 mg/ml) for 20 min and insulin release measured by RIA. Values are expressed as mean \pm S.E.M. (n=8). **p<0.01, ***p<0.001 compared to respective glucose control.

Fig 3.11

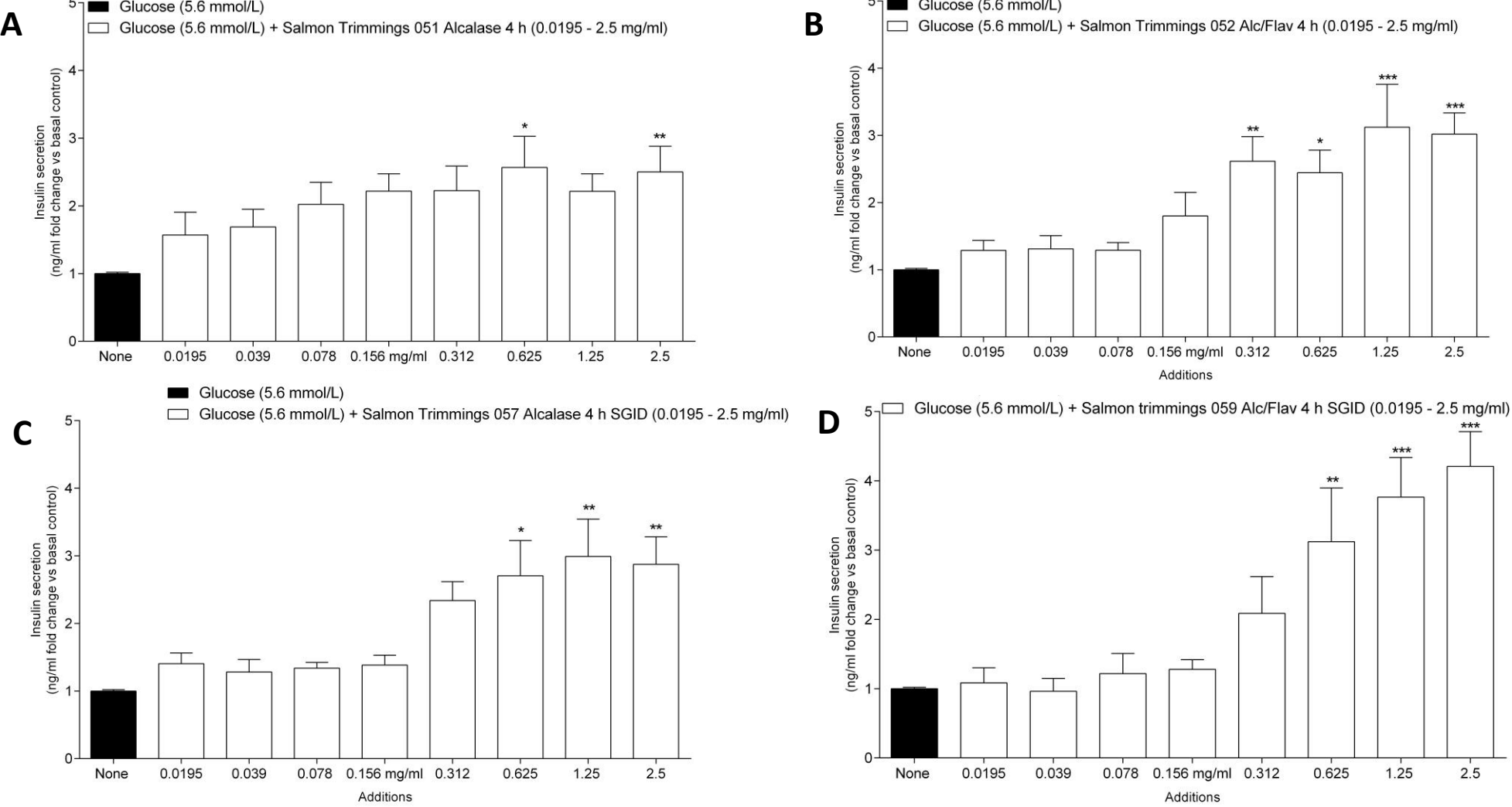


Fig 3.11. Dose-dependent effects of Salmon trimming protein hydrolysates on insulin secretion from BRIN-BD11 cells at 5.6 mM glucose. BRIN-BD11 cells were incubated with either ULPH051 (**Panel A**), ULPH052 (**Panel B**), ULPH057 (**Panel C**) or ULPH059 (**Panel D**) using a range of concentrations (2.5 to 0.0195 mg/ml) for 20 min and insulin release measured by RIA. Values are expressed as mean \pm S.E.M. (n=8). **p<0.01, ***p<0.001 compared to respective glucose control.

Fig 3.12

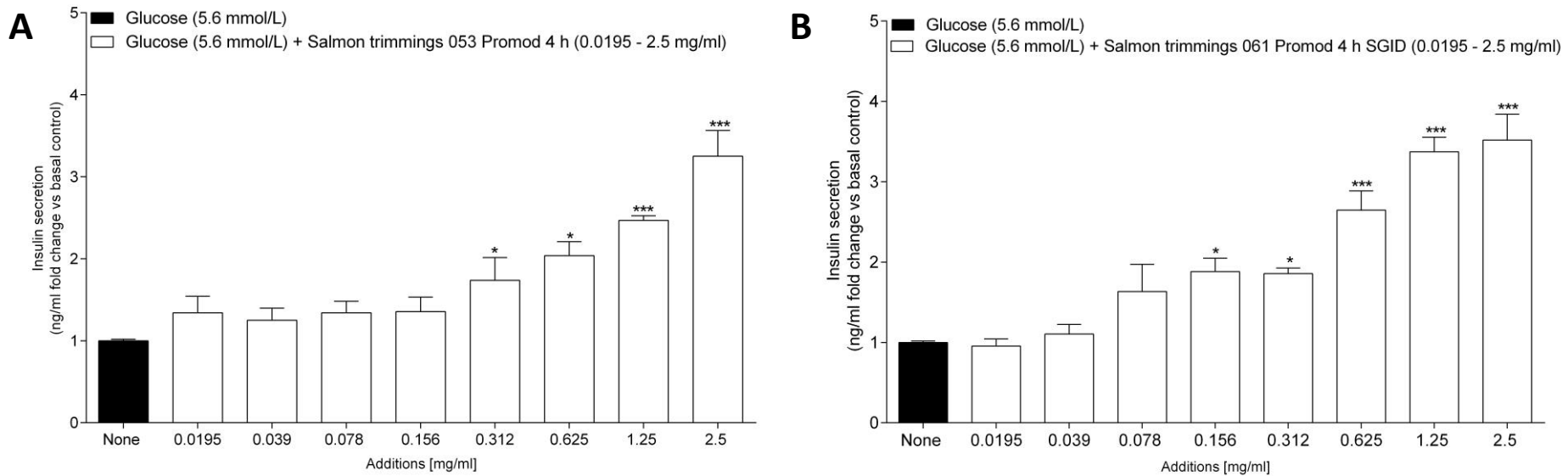


Fig 3.12 Dose-dependent effects of Salmon trimming protein hydrolysates on insulin secretion from BRIN-BD11 cells at 5.6 mM glucose. BRIN-BD11 cells were incubated with either ULPH053 (**Panel A**) or ULPH061 (**Panel B**) using a range of concentrations (2.5 to 0.0195 mg/ml) for 20 min and insulin release measured by RIA. Values are expressed as mean \pm S.E.M. (n=8). **p<0.01, ***p<0.001 compared to respective glucose control.

Fig 3.13

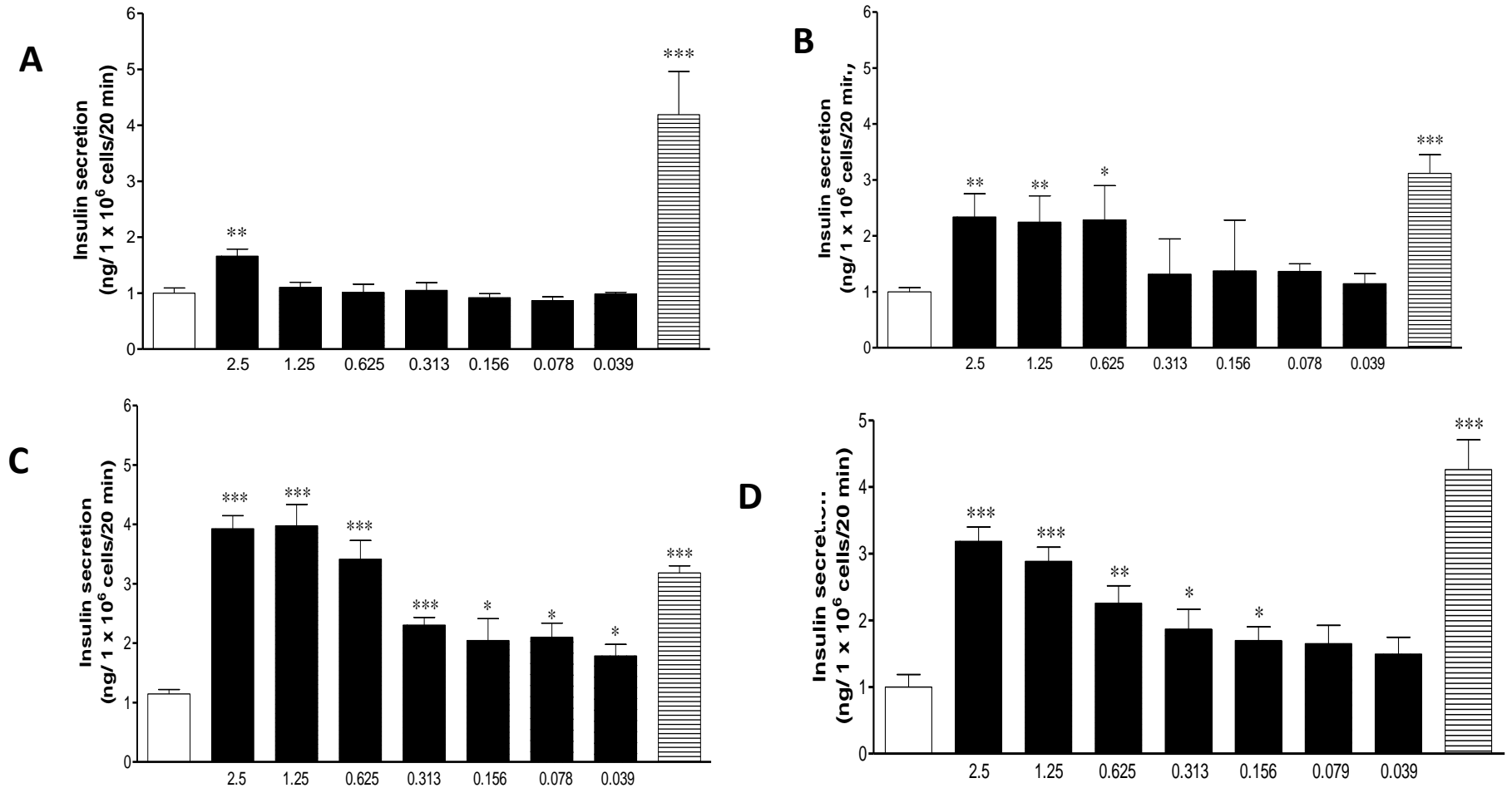


Fig 3.13. Dose-dependent effects of Salmon trimming protein hydrolysates on insulin secretion from BRIN-BD11 cells at 5.6 mM glucose. BRIN-BD11 cells were incubated with either ULPH053 (**Panel A**) or ULPH061 (**Panel B**) using a range of concentrations (2.5 to 0.0195 mg/ml) for 20 min and insulin release measured by RIA. Values are expressed as mean \pm S.E.M. (n=8). **p<0.01, ***p<0.001 compared to respective glucose control.

Table 1 – BRIN-BD11 cell summary

Fold increase insulin secretion summary of BRIN-BD11				
Hydrolysate	Source	Glucose/Hydrolysate	Fold increase at max concentration	Lowest activity (mg/ml)
18	P.palmata	5.6 mM / 2.5-0.039 mg/ml	2.6	1.25, **p<0.01
		16.7 mM / 2.5-0.039 mg/ml	2.5	0.312, **p<0.01
42	P.palmata	5.6 mM / 2.5-0.039 mg/ml	3.4	0.312, *p<0.05
		16.7 mM / 2.5-0.039 mg/ml	4.6	0.156, **p<0.01
19	P.palmata	5.6 mM / 2.5-0.039 mg/ml	3.9	0.312, ***p<0.001
		16.7 mM / 2.5-0.039 mg/ml	4.4	0.625, ***p<0.001
34	P.palmata	5.6 mM / 2.5-0.039 mg/ml	2.1	1.25, *p<0.05
		16.7 mM / 2.5-0.039 mg/ml	3.1	1.25, ***p<0.001
20	P.palmata	5.6 mM / 2.5-0.039 mg/ml	4	0.156, **p<0.01
		16.7 mM / 2.5-0.039 mg/ml	4.1	0.312, **p<0.01
36	P.palmata	5.6 mM / 2.5-0.039 mg/ml	3.6	0.625, ***p<0.001
		16.7 mM / 2.5-0.039 mg/ml	3.9	0.625, ***p<0.001
21	P.palmata	5.6 mM / 2.5-0.039 mg/ml	4.3	0.078, *p<0.05
		16.7 mM / 2.5-0.039 mg/ml	4.2	0.625, *p<0.05
38	P.palmata	5.6 mM / 2.5-0.039 mg/ml	3.6	1.25, ***p<0.001
		16.7 mM / 2.5-0.039 mg/ml	3.8	0.312, **p<0.01
22	P.palmata	5.6 mM / 2.5-0.039 mg/ml	3.2	0.625, **p<0.01
		16.7 mM / 2.5-0.039 mg/ml	4.2	0.625, ***p<0.001
40	P.palmata	5.6 mM / 2.5-0.039 mg/ml	3.2	0.625, *p<0.05
		16.7 mM / 2.5-0.039 mg/ml	4.3	0.156, ***p<0.001
Hydrolysate	Source	Glucose/Hydrolysate	Fold increase at max concentration	Lowest activity (mg/ml)
63	Blue whiting	5.6 mM / 2.5-0.039 mg/ml	3.8	0.039, ***p<0.001
70	Blue whiting	5.6 mM / 2.5-0.039 mg/ml	4.2	0.078, **p<0.01
72	Blue whiting	5.6 mM / 2.5-0.039 mg/ml	4.1	0.078, ***p<0.001
Hydrolysate	Source	Glucose/Hydrolysate	Fold increase at max concentration	Lowest activity (mg/ml)
62	Boarfish	5.6 mM / 2.5-0.039 mg/ml	4.1	0.078, **p<0.01
68	Boarfish	5.6 mM / 2.5-0.039 mg/ml	5.4	0.156, **p<0.01
66	Boarfish	5.6 mM / 2.5-0.039 mg/ml	5.3	0.156, **p<0.01
74	Boarfish	5.6 mM / 2.5-0.039 mg/ml	4.5	0.156, **p<0.01
		16.7 mM / 2.5-0.039 mg/ml	3.4	0.312, ***p<0.001
Hydrolysate	Source	Glucose/Hydrolysate	Fold increase at max concentration	Lowest activity (mg/ml)
51	Salmon trimming	5.6 mM / 2.5-0.039 mg/ml	2.5	0.625, *p<0.05
57	Salmon trimming	5.6 mM / 2.5-0.039 mg/ml	2.8	0.625, *p<0.05
52	Salmon trimming	5.6 mM / 2.5-0.039 mg/ml	3	0.312, **p<0.01
59	Salmon trimming	5.6 mM / 2.5-0.039 mg/ml	4.2	0.625, *p<0.05
53	Salmon trimming	5.6 mM / 2.5-0.039 mg/ml	3.2	0.312, *p<0.05
61	Salmon trimming	5.6 mM / 2.5-0.039 mg/ml	3.5	0.156, p<0.05
Hydrolysate	Source	Glucose/Hydrolysate	Fold increase at max concentration	Lowest activity (mg/ml)
32	Salmon gelatine	5.6 mM / 2.5-0.039 mg/ml	1.8	2.5, **p<0.01
24	Salmon gelatine	5.6 mM / 2.5-0.039 mg/ml	2.5	0.625, *p<0.05
26	Salmon gelatine	5.6 mM / 2.5-0.039 mg/ml	3.9	0.039, *p<0.05
28	Salmon gelatine	5.6 mM / 2.5-0.039 mg/ml	3.1	0.156, p<0.05

Fig 3.14

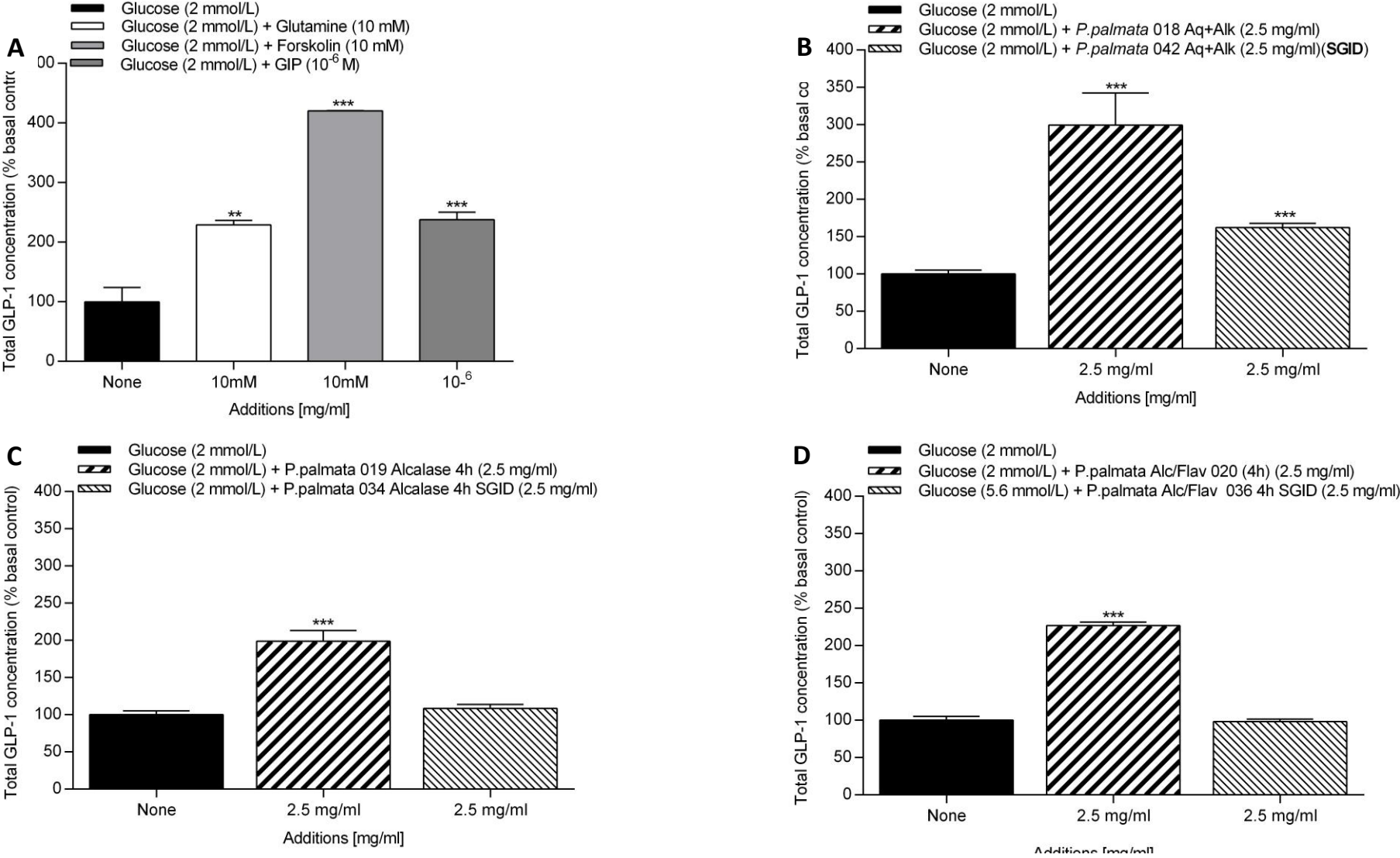


Fig 3.14. Secretory effects of incretin secreting hydrolysates on GLP-1 secretion from GLUTag cells at 2 mM glucose. GLUTag cells were incubated with a range of incretin secreting positive controls (**Panel A**), ULPH018/042 (**Panel B**), ULPH19/034 (**Panel C**) or ULPH020/036 using a fixed concentrations of hydrolysate (2.5 mg/ml) for 2 hours and GLP-1 release measured by ELISA. Values are expressed as mean \pm S.E.M. (n=3). **p<0.01, ***p<0.001 compared to respective glucose control.

Fig 3.15

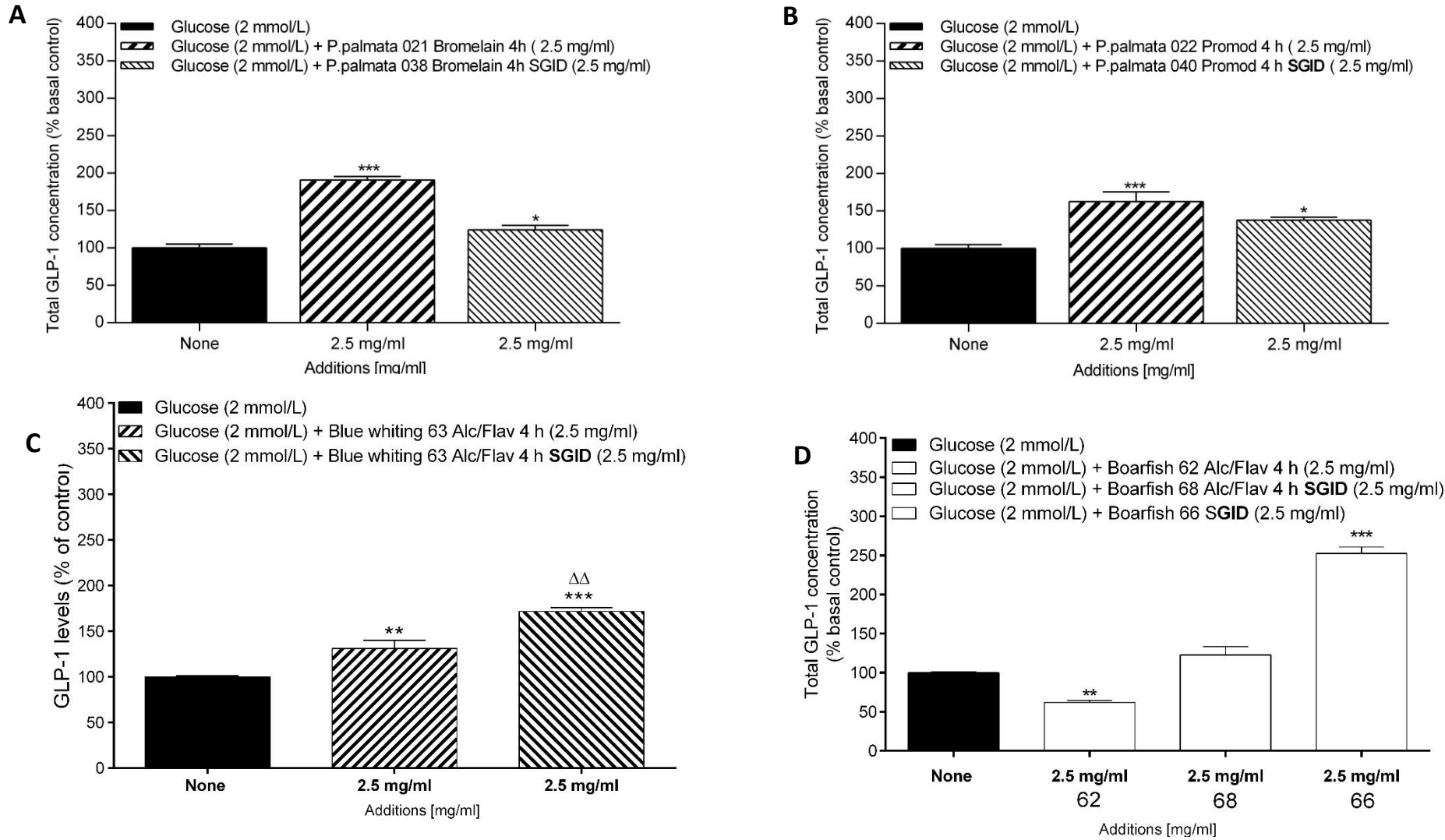


Fig 3.15. Effects of GLP-1 secreting marine protein hydrolysates from GLUTag cells at 2 mM glucose. GLUTag cells were co-incubated with ULPH021/038 (**Panel A**), ULPH022/040 (**Panel B**), ULPH063 (**Panel C**) or ULPH062/068/066 (**Panel D**) using a fixed concentrations of hydrolysate (2.5 mg/ml) for 2 hours and GLP-1 release measured by ELISA. Values are expressed as mean ± S.E.M. (n=3). *p<0.05, **p<0.01, ***p<0.001 compared to respective glucose control.

Fig 3.16

A

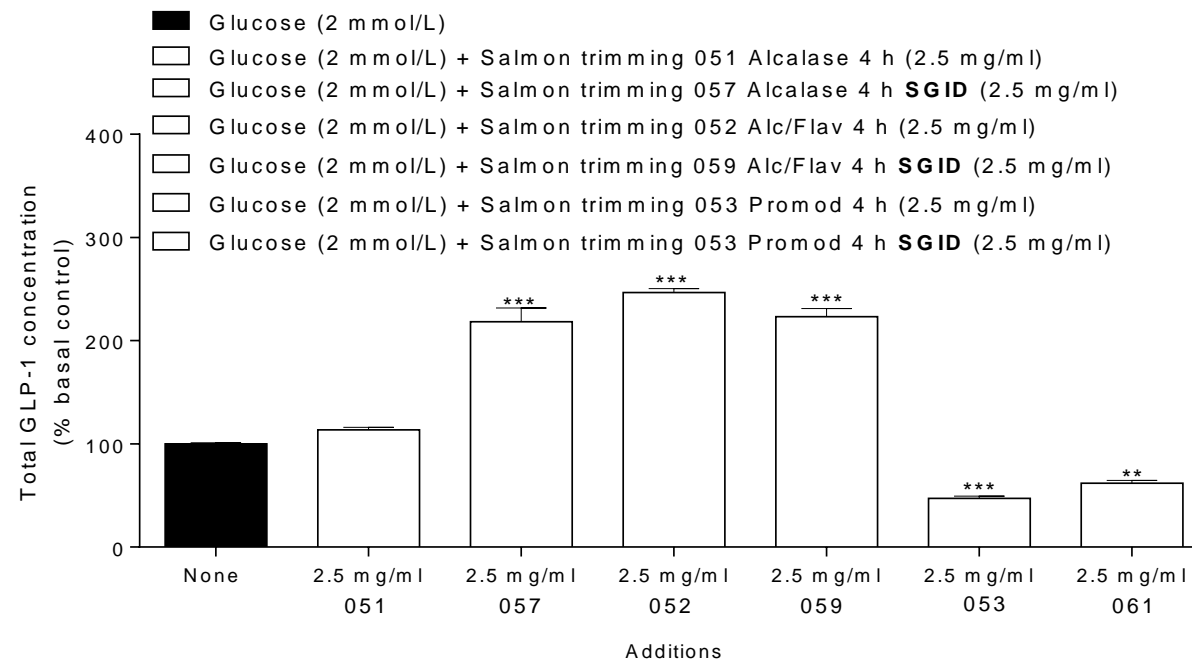


Fig 3.16. Effects of GLP-1 secreting marine protein hydrolysates from GLUTag cells at 2 mM glucose. GLUTag cells were co-incubated with ULPH051/057/052/059 or 053 (**Panel A**), using a fixed concentrations of hydrolysate (2.5 mg/ml) for 2 hours and GLP-1 release measured by ELISA. Values are expressed as mean \pm S.E.M. (n=3). **p<0.01,***p<0.001 compared to respective glucose control.

Table 2 – GLUTag cell summary

Fold increase of GLP-1 secretion summary of GLUTag cells					
Hydrolysate	Source	Glucose/hydrolysate	Fold increase at max concentration	Significance	SGID
18	<i>P.palmata</i>	2 mM / 2.5 mg/ml	3	***p<0.001	n
42	<i>P.palmata</i>	2 mM / 2.5 mg/ml	1.6	***p<0.001	y
19	<i>P.palmata</i>	2 mM / 2.5 mg/ml	2	***p<0.001	n
34	<i>P.palmata</i>	2 mM / 2.5 mg/ml	0	ns	y
20	<i>P.palmata</i>	2 mM / 2.5 mg/ml	2.2	***p<0.001	n
36	<i>P.palmata</i>	2 mM / 2.5 mg/ml	0	ns	y
21	<i>P.palmata</i>	2 mM / 2.5 mg/ml	1.9	***p<0.001	n
38	<i>P.palmata</i>	2 mM / 2.5 mg/ml	1.2	*p<0.05	y
22	<i>P.palmata</i>	2 mM / 2.5 mg/ml	1.6	***p<0.001	n
40	<i>P.palmata</i>	2 mM / 2.5 mg/ml	1.3	*p<0.05	y
63	Blue whiting	2 mM / 2.5 mg/ml	1.31	**p<0.01	n
70	Blue whiting	2 mM / 2.5 mg/ml	1.71	***p<0.001	y
62	Boarfish	2 mM / 2.5 mg/ml	-0.4	**p<0.01	n
68	Boarfish	2 mM / 2.5 mg/ml	0	ns	y
66	Boarfish	2 mM / 2.5 mg/ml	2.5	***p<0.001	y
51	Salmon Trimming	2 mM / 2.5 mg/ml	0	ns	n
57	Salmon Trimming	2 mM / 2.5 mg/ml	2.1	***p<0.001	y
52	Salmon Trimming	2 mM / 2.5 mg/ml	2.4	***p<0.001	n
59	Salmon Trimming	2 mM / 2.5 mg/ml	2.2	***p<0.001	y
53	Salmon Trimming	2 mM / 2.5 mg/ml	-0.5	***p<0.001	n
61	Salmon Trimming	2 mM / 2.5 mg/ml	-0.4	**p<0.01	y
32	Salmon gelatine	2 mM / 2.5 mg/ml	2	***p<0.001	y
24	Salmon gelatine	2 mM / 2.5 mg/ml	3	***p<0.001	y
26	Salmon gelatine	2 mM / 2.5 mg/ml	3.4	***p<0.001	y
28	Salmon gelatine	2 mM / 2.5 mg/ml	3.8	***p<0.001	y

3.17

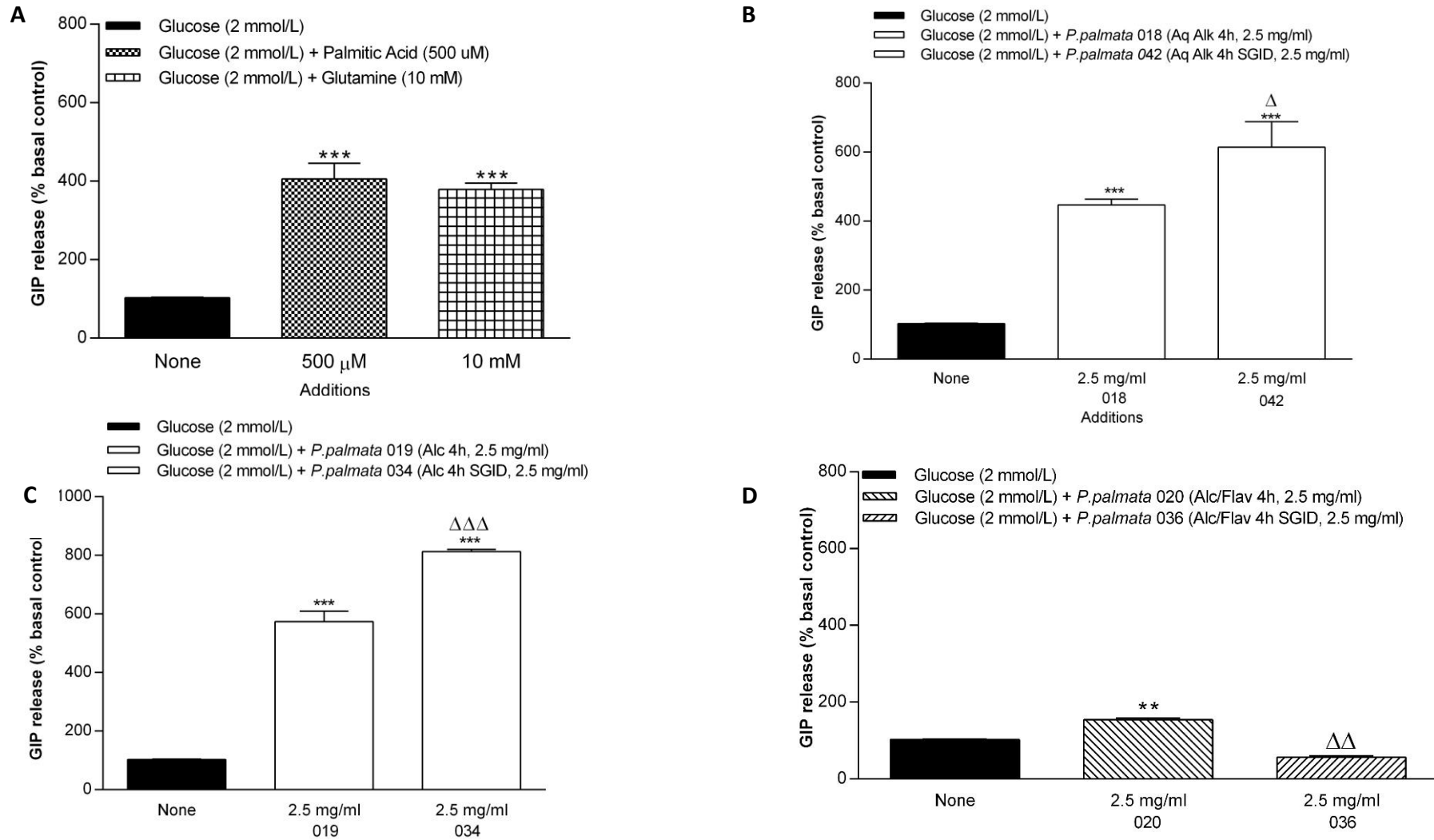


Fig 3.17. Secretory effects of incretin secreting hydrolysates on GIP secretion from STC-1 cells at 2 mM glucose. STC-1 cells were incubated with a range of incretin secreting positive controls (**Panel A**), ULPH018/042 (**Panel B**), ULPH19/034 (**Panel C**) or (**Panel D**) ULPH020/036 using a fixed concentrations of hydrolysate (2.5 mg/ml) for 2 hours and GLP-1 release measured by ELISA. Values are expressed as mean \pm S.E.M. (n=3). **p<0.01, ***p<0.001 compared to respective glucose control.

3.18

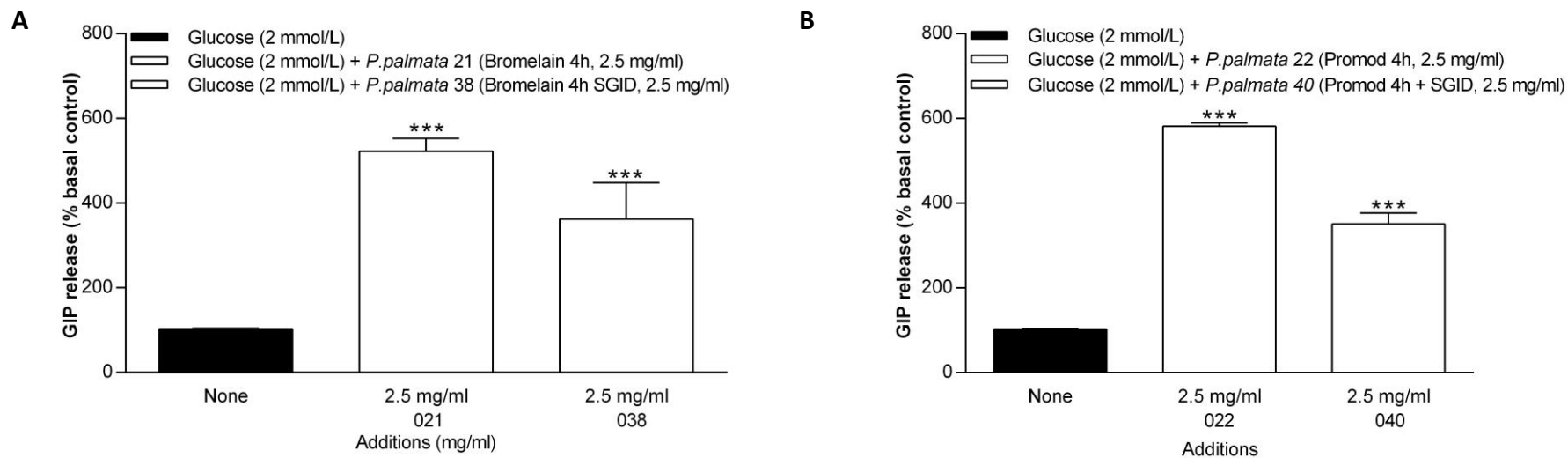


Fig 3.18. Effects of GIP secreting marine protein hydrolysates from STC-1 cells at 2 mM glucose. STC-1 cells were co-incubated with ULPH021/038 (**Panel A**) or ULPH022/040 (**Panel B**) using a fixed concentrations of hydrolysate (2.5 mg/ml) for 2 hours and GLP-1 release measured by ELISA. Values are expressed as mean \pm S.E.M. (n=3). *p<0.05, **p<0.01, ***p<0.001 compared to respective glucose control.

3.19

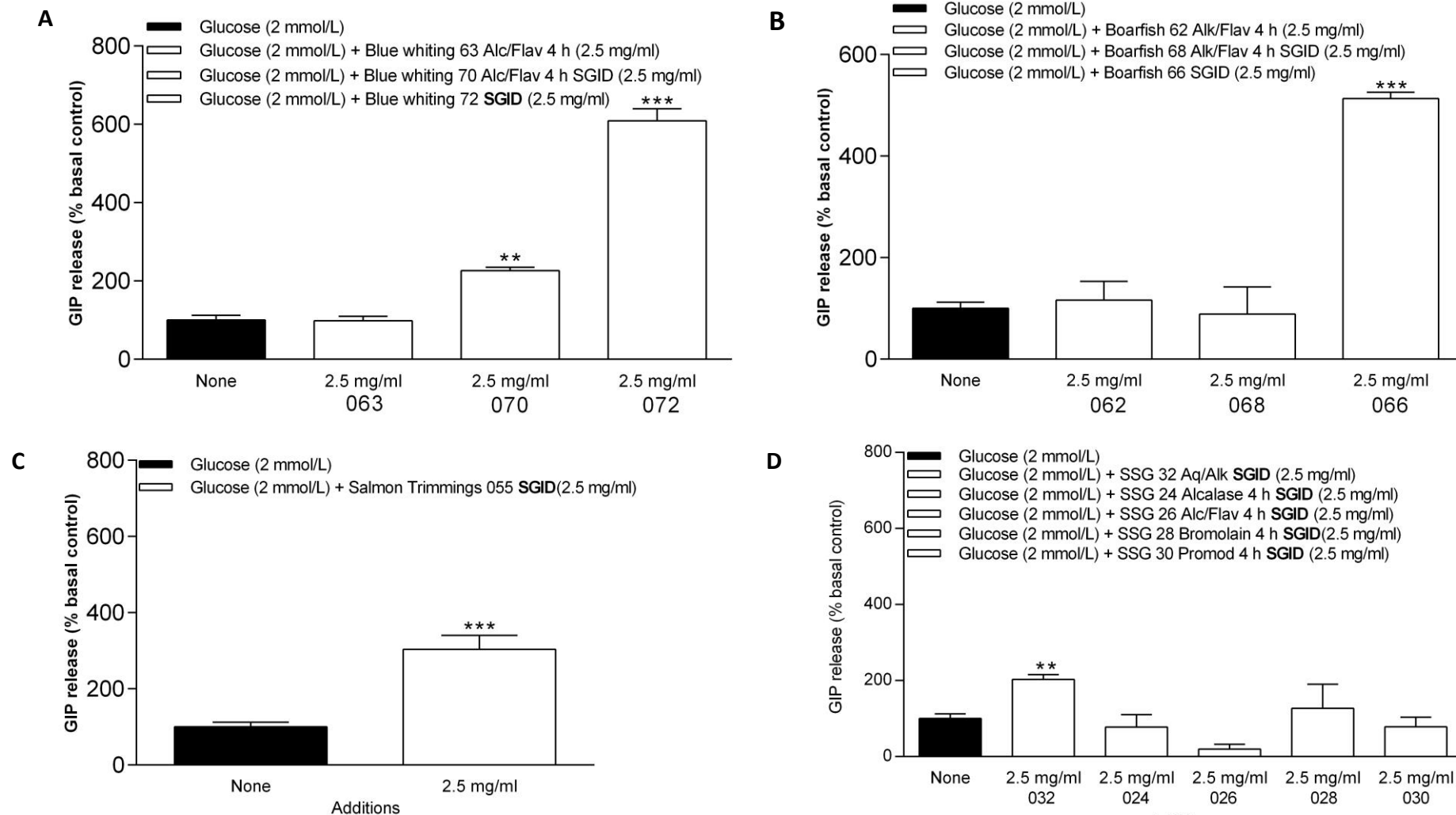


Fig 3.19. Effects of incretin secreting hydrolysates on GIP secretion from STC-1 cells at 2 mM glucose. STC-1 cells were incubated with either blue whiting ULPH063/070/072 (**Panel A**), Boarfish ULPH0062/068/066 (**Panel B**), Salmon trimmings ULPH055 (**Panel C**) or Salmon skin gelatin ULPH032/024/026/028/030 (**Panel D**) using a fixed concentrations of hydrolysate (2.5 mg/ml) for 2 hours and GLP-1 release measured by ELISA. Values are expressed as mean \pm S.E.M. (n=3). **p<0.01, ***p<0.001 compared to respective glucose control.

Table 3 – STC-1 cell summary

Fold increase of GIP secretion summary of STC-1 cells					
Hydrolysate	Source	Glucose/Hydrolysate	Fold increase at max concentration	Significance	SGID
18	<i>P.palmata</i>	2 mM / 2.5 mg/ml	4.4	***p<0.001	n
42	<i>P.palmata</i>	2 mM / 2.5 mg/ml	6.1	***p<0.001	y
19	<i>P.palmata</i>	2 mM / 2.5 mg/ml	5.7	***p<0.001	n
34	<i>P.palmata</i>	2 mM / 2.5 mg/ml	8.1	***p<0.001	y
20	<i>P.palmata</i>	2 mM / 2.5 mg/ml	1.5	**p<0.01	n
36	<i>P.palmata</i>	2 mM / 2.5 mg/ml	-0.5	*p<0.05	y
21	<i>P.palmata</i>	2 mM / 2.5 mg/ml	5.2	***p<0.001	n
38	<i>P.palmata</i>	2 mM / 2.5 mg/ml	3.6	***p<0.001	y
22	<i>P.palmata</i>	2 mM / 2.5 mg/ml	5.8	***p<0.001	n
40	<i>P.palmata</i>	2 mM / 2.5 mg/ml	3.5	***p<0.001	y
63	Blue whiting	2 mM / 2.5 mg/ml	0	ns	n
70	Blue whiting	2 mM / 2.5 mg/ml	2.2	***p<0.001	y
72	Blue whiting	2 mM / 2.5 mg/ml	6	***p<0.001	y
62	Boarfish	2 mM / 2.5 mg/ml	0	ns	n
68	Boarfish	2 mM / 2.5 mg/ml	0	ns	y
66	Boarfish	2 mM / 2.5 mg/ml	5.1	***p<0.001	y
55	Salmon Trimming	2 mM / 2.5 mg/ml	3	***p<0.001	n
32	Salmon gelatine	2 mM / 2.5 mg/ml	2	**p<0.01	y
24	Salmon gelatine	2 mM / 2.5 mg/ml	0	ns	y
26	Salmon gelatine	2 mM / 2.5 mg/ml	0	ns	y
28	Salmon gelatine	2 mM / 2.5 mg/ml	0	ns	y
30	Salmon gelatine	2 mM / 2.5 mg/ml	0	ns	y

3.20

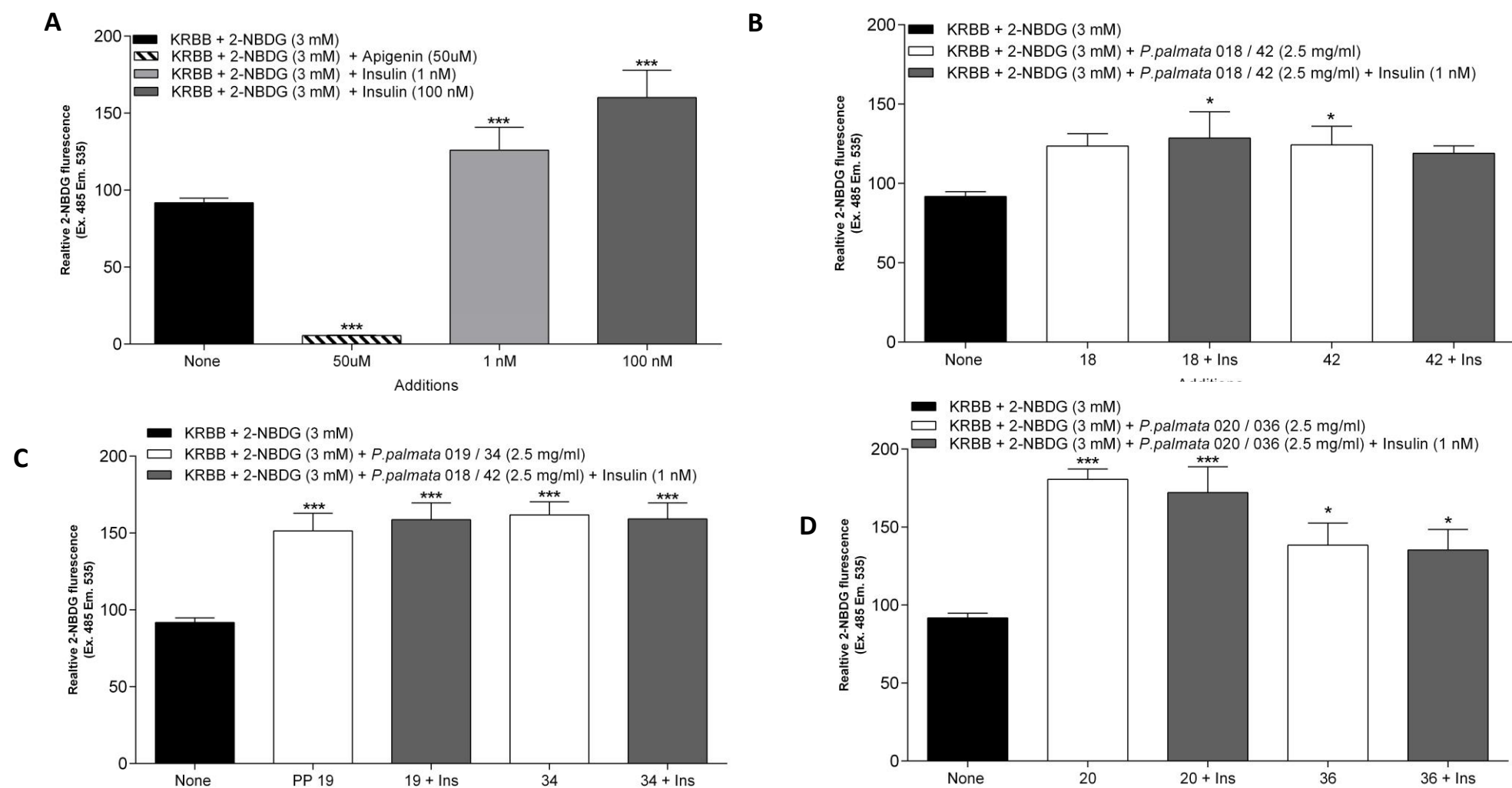


Fig 3.20. Effects of marine protein hydrolysates on glucose uptake individually, or in combination with insulin in trans-differentiated 3T3-L1 adipocyte cells. Adipocyte cells were incubated with either Apigenin (50 μ M), Insulin (1 nM or 100 nM) (**Panel A**), ULPH018 or 042 (with or without 1 nM insulin, (**Panel B**), ULPH019 or 034 (With or without 1 nM insulin, (**Panel C**) or ULPH020 or 036 (with or without 1 nM insulin, (**Panel D**) using a fixed concentrations of hydrolysate (2.5 mg/ml) for 1 hour with 3 mM fluorescent glucose. Values are expressed as mean \pm S.E.M. (n=3). $8p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to respective glucose control.

3.21

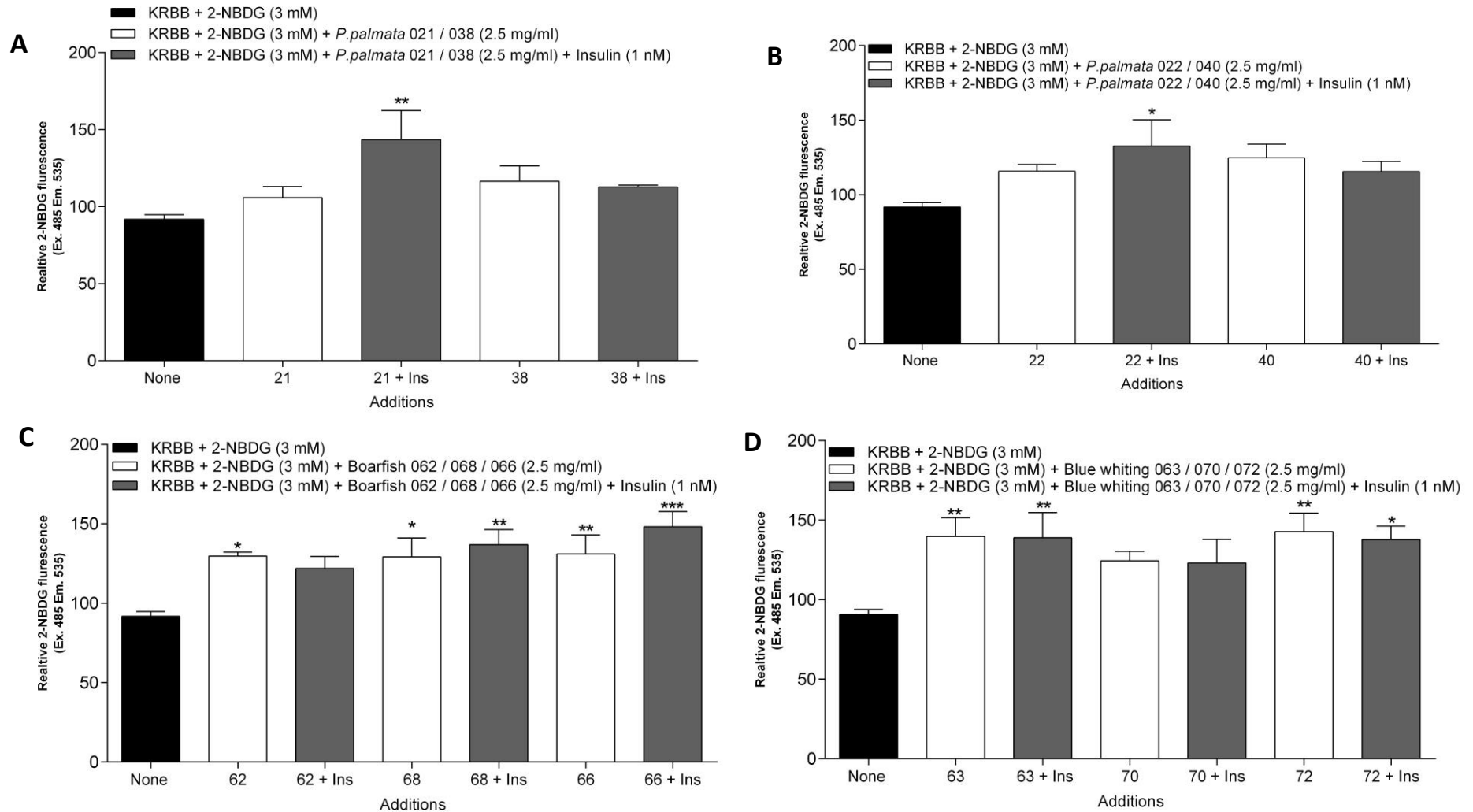


Fig 3.21. Effects of marine protein hydrolysates on glucose uptake individually, or in combination with insulin in trans-differentiated 3T3-L1 adipocyte cells. Adipocyte cells were incubated with either ULPH021 or 038 (With or without 1 nM insulin, **Panel A**), ULPH022 or 040 (with or without 1 nM insulin, **Panel B**), ULPH062, 068 or 066 (With or without 1nM insulin, **Panel C**) or ULPH063 or 070 or 072 (with or without 1 nM insulin, **Panel D**) using a fixed concentrations of hydrolysate (2.5 mg/ml) for 1 hour with 3 mM fluorescent glucose (2-NBDG). Values are expressed as mean \pm S.E.M. (n=3). $^{\#}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to respective glucose control.

Table 4 – Glucose uptake summary

Glucose (2-NBDG) uptake activity in 3T3-L1 cells summary					
Hydrolysate	Source	2-NBDG/Hydrolysate/Insulin	Increased glucose uptake %	Significance	SGID
Control	Apigenin (50 uM)	3 mM / 0 mg/ml / 0	-95%	***p<0.001	
18	<i>P.palmata</i>	3 mM / 2.5 mg/ml / 0	23%	ns	n
18 + Insulin		3 mM / 2.5 mg/ml / 1 nM	28%	*p<0.05	
42	<i>P.palmata</i>	3 mM / 2.5 mg/ml / 0	24%	*p<0.05	y
42 + Insulin		3 mM / 2.5 mg/ml / 1 nM	18%	ns	
19	<i>P.palmata</i>	3 mM / 2.5 mg/ml / 0	51%	***p<0.001	n
19 + Insulin		3 mM / 2.5 mg/ml / 1 nM	58%	***p<0.001	
34	<i>P.palmata</i>	3 mM / 2.5 mg/ml / 0	61%	***p<0.001	y
34 + Insulin		3 mM / 2.5 mg/ml / 1 nM	59%	***p<0.001	
20	<i>P.palmata</i>	3 mM / 2.5 mg/ml / 0	80%	***p<0.001	n
20 + Insulin		3 mM / 2.5 mg/ml / 1 nM	72%	***p<0.001	
36	<i>P.palmata</i>	3 mM / 2.5 mg/ml / 0	38%	*p<0.05	y
36 + Insulin		3 mM / 2.5 mg/ml / 1 nM	35%	*p<0.05	
21	<i>P.palmata</i>	3 mM / 2.5 mg/ml / 0	5%	ns	n
21 + Insulin		3 mM / 2.5 mg/ml / 1 nM	43%	**p<0.01	
38	<i>P.palmata</i>	3 mM / 2.5 mg/ml / 0	16%	ns	y
38 + Insulin		3 mM / 2.5 mg/ml / 1 nM	12%	ns	
22	<i>P.palmata</i>	3 mM / 2.5 mg/ml / 0	15%	ns	n
22 + Insulin		3 mM / 2.5 mg/ml / 1 nM	32%	*p<0.05	
40	<i>P.palmata</i>	3 mM / 2.5 mg/ml / 0	24%	ns	y
40 + Insulin		3 mM / 2.5 mg/ml / 1 nM	15%	ns	
63	Blue whiting	3 mM / 2.5 mg/ml / 0	39%	**p<0.01	n
63 + Insulin		3 mM / 2.5 mg/ml / 1 nM	38%	**p<0.01	
70	Blue whiting	3 mM / 2.5 mg/ml / 0	24%	ns	y
70 + Insulin		3 mM / 2.5 mg/ml / 1 nM	23%	ns	
72	Blue whiting	3 mM / 2.5 mg/ml / 0	42%	**p<0.01	y
72 + Insulin		3 mM / 2.5 mg/ml / 1 nM	37%	*p<0.05	
62	Boarfish	3 mM / 2.5 mg/ml / 0	29%	*p<0.05	n
62 + Insulin		3 mM / 2.5 mg/ml / 1 nM	21%	ns	
68	Boarfish	3 mM / 2.5 mg/ml / 0	29%	*p<0.05	y
68 + Insulin		3 mM / 2.5 mg/ml / 1 nM	36%	**p<0.01	
66	Boarfish	3 mM / 2.5 mg/ml / 0	31%	**p<0.01	y
66 + Insulin		3 mM / 2.5 mg/ml / 1 nM	48%	***p<0.001	

3.22

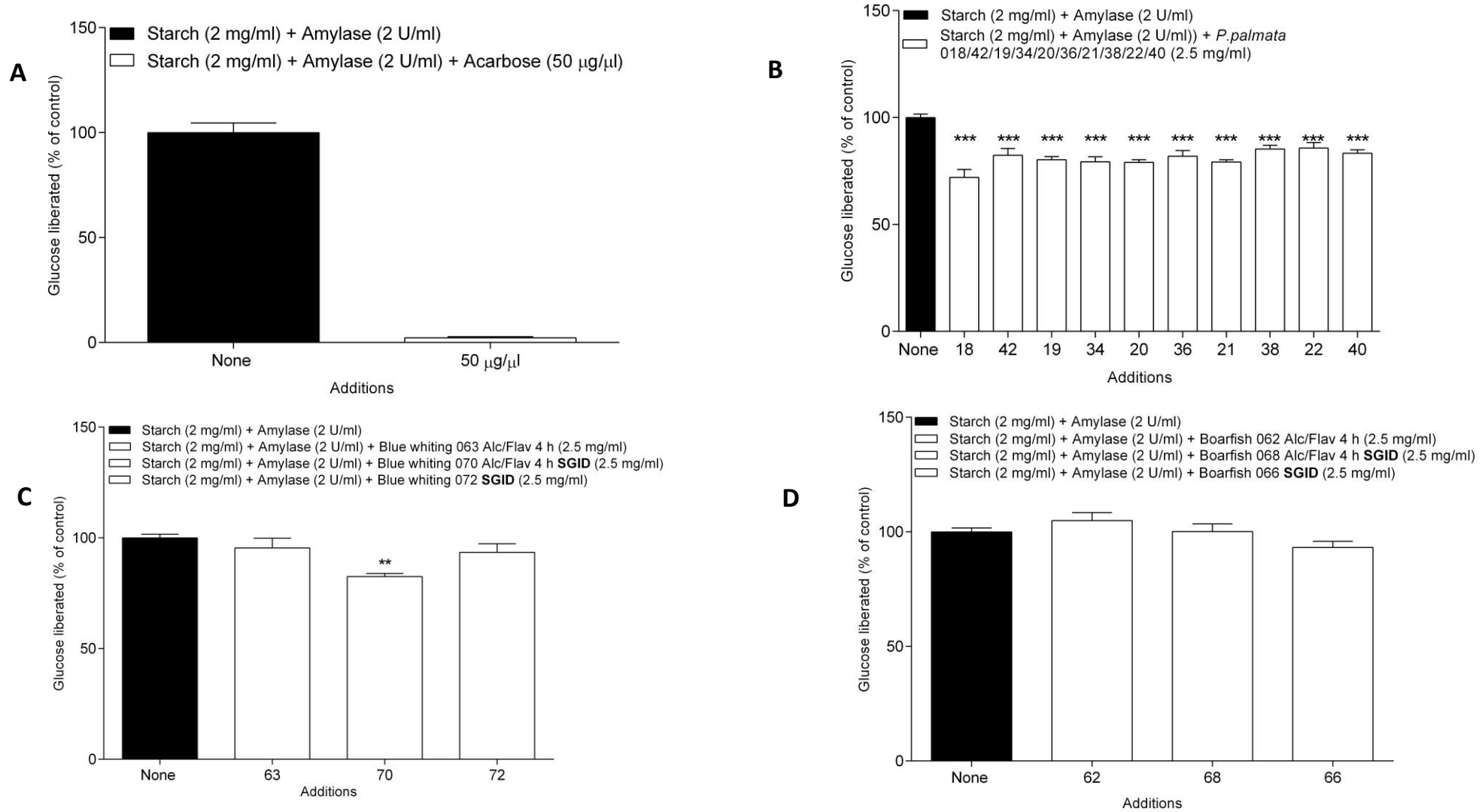


Fig 3.22. Effects of marine protein hydrolysates on amylase activity in the presence of starch. Starch (2 mg/ml) was incubated with Amylase (2 U/ml) with either Acarbose (**Panel A**), ULPH018, 042, 019, 034, 020, 036, 021, 038, 022 or 040 (**Panel B**), ULPH063, 070 or 072 (**Panel C**) or ULPH062 or 068 or 066 (**Panel D**) using a fixed concentrations of hydrolysate (2.5 mg/ml) for 1 hour with liberated glucose analysed using the GODPAP assay. Values are expressed as mean ± S.E.M. (n=3). **p<0.01, ***p<0.001 compared to respective amylase only control.

3.23

A

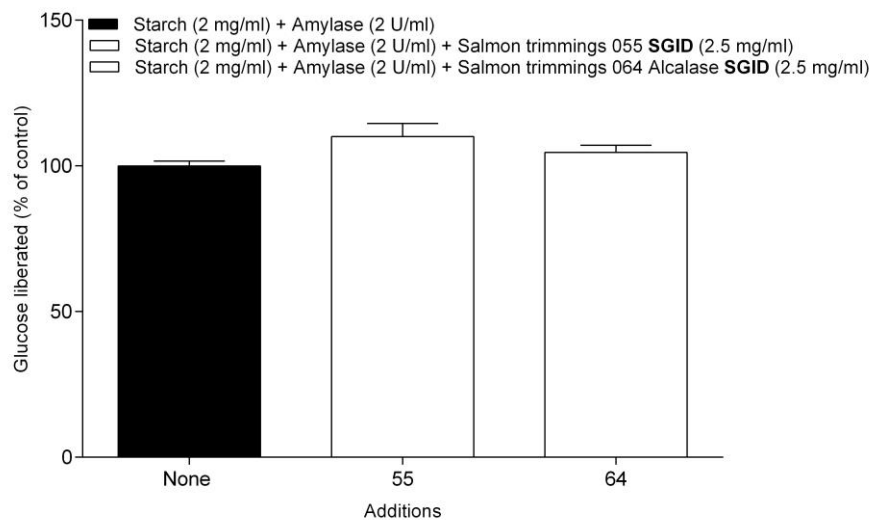


Fig 3.23. Effects of marine protein hydrolysates on amylase activity in the presence of starch. Starch (2 mg/ml) was incubated with Amylase (2 U/ml) only or ULPH055 or ULPH064 (**Panel A**), using a fixed concentration of hydrolysate (2.5 mg/ml) for 1 hour with liberated glucose analysed using the GODPAP assay. Values are expressed as mean \pm S.E.M. (n=3) compared to respective amylase only control.

Table 5 – Amylase activity summary

Glucose liberation via Amylase activity summary					
Hydrolysate	Source	Amylase/Hydrolysate	% Amylase activity decrease	Significance	SGID
None	Apigenin	2U / 0 mg/ml	95%	***p<0.001	
18	<i>P.palmata</i>	2U / 2.5 mg/ml	28%	***p<0.001	n
42	<i>P.palmata</i>	2U / 2.5 mg/ml	18%	***p<0.001	y
19	<i>P.palmata</i>	2U / 2.5 mg/ml	20%	***p<0.001	n
34	<i>P.palmata</i>	2U / 2.5 mg/ml	21%	***p<0.001	y
20	<i>P.palmata</i>	2U / 2.5 mg/ml	21%	***p<0.001	n
36	<i>P.palmata</i>	2U / 2.5 mg/ml	19%	***p<0.001	y
21	<i>P.palmata</i>	2U / 2.5 mg/ml	21%	***p<0.001	n
38	<i>P.palmata</i>	2U / 2.5 mg/ml	15%	***p<0.001	y
22	<i>P.palmata</i>	2U / 2.5 mg/ml	15%	***p<0.001	n
40	<i>P.palmata</i>	2U / 2.5 mg/ml	18%	***p<0.001	y
63	Blue whiting	2U / 2.5 mg/ml	5%	ns	n
70	Blue whiting	2U / 2.5 mg/ml	18%	***p<0.001	y
72	Blue whiting	2U / 2.5 mg/ml	7%	ns	y
62	Boarfish	2U / 2.5 mg/ml	-4%	ns	n
68	Boarfish	2U / 2.5 mg/ml	0%	ns	y
66	Boarfish	2U / 2.5 mg/ml	7%	ns	y
55	Salmon Trimming	2U / 2.5 mg/ml	-10%	ns	n
64	Salmon Trimming	2U / 2.5 mg/ml	-4%	ns	y

3.24

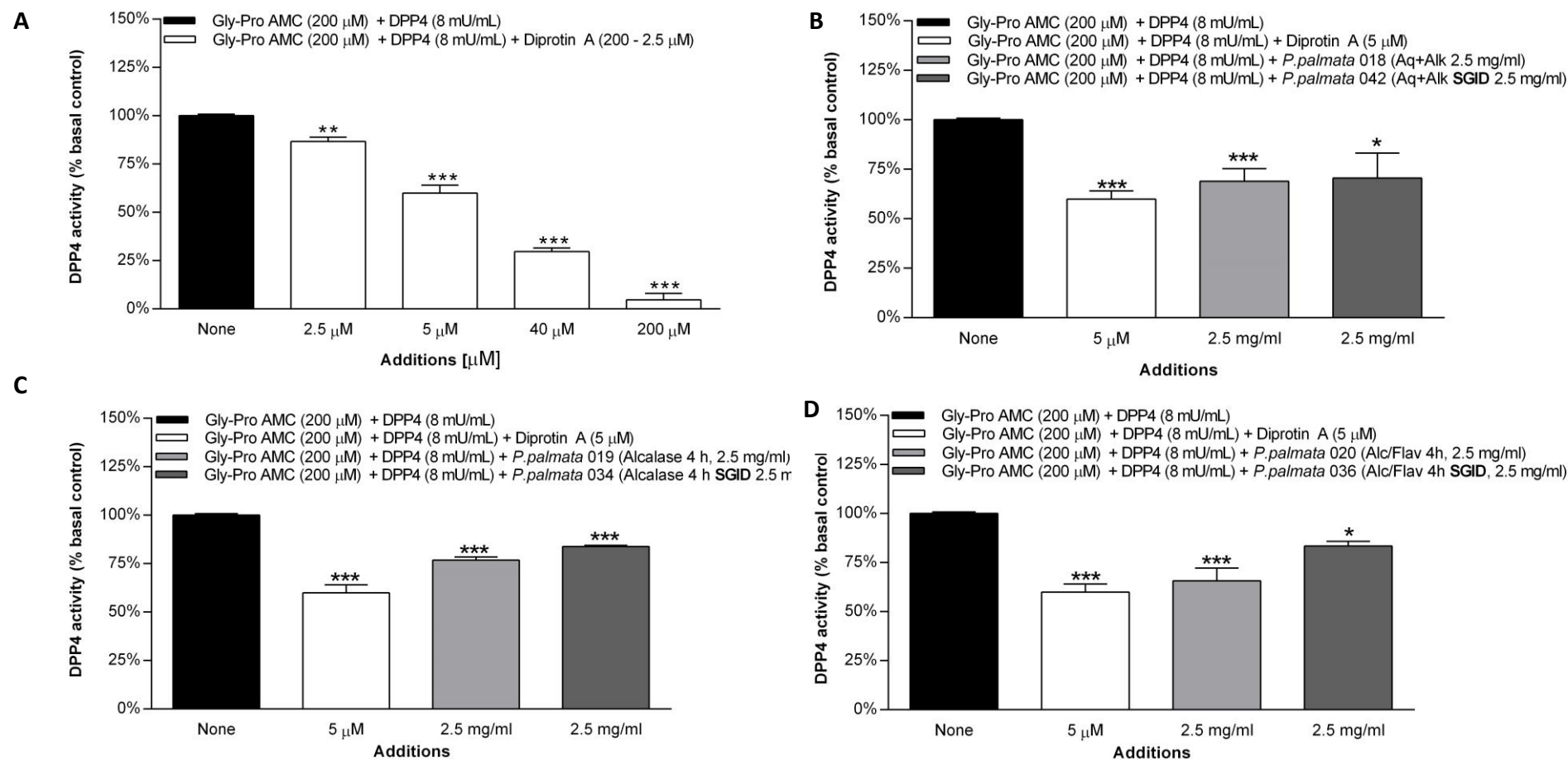


Fig 3.24. Effects of marine protein hydrolysates on DPP4 activity and liberation of the fluorogenic AMC substrate. Gly-Pro-AMC (200 μM) was incubated with DPP4 (8 mU/ml) alone or with either a dose dependant concentration of Diprotin A (2.5 – 200 μM , **Panel A**), ULPH018 or 042 (**Panel B**), ULPH019 or 034 (**Panel C**) or ULPH020 or 036 (**Panel D**) using a fixed concentrations of hydrolysate (2.5 mg/ml) for 30 min with liberated AMC analysed using EX/EM 360/460 nm wavelength. Values are expressed as mean \pm S.E.M. (n=3). *p<0.05, **p<0.01, ***p<0.001 compared to respective DPP4 only control.

3.25

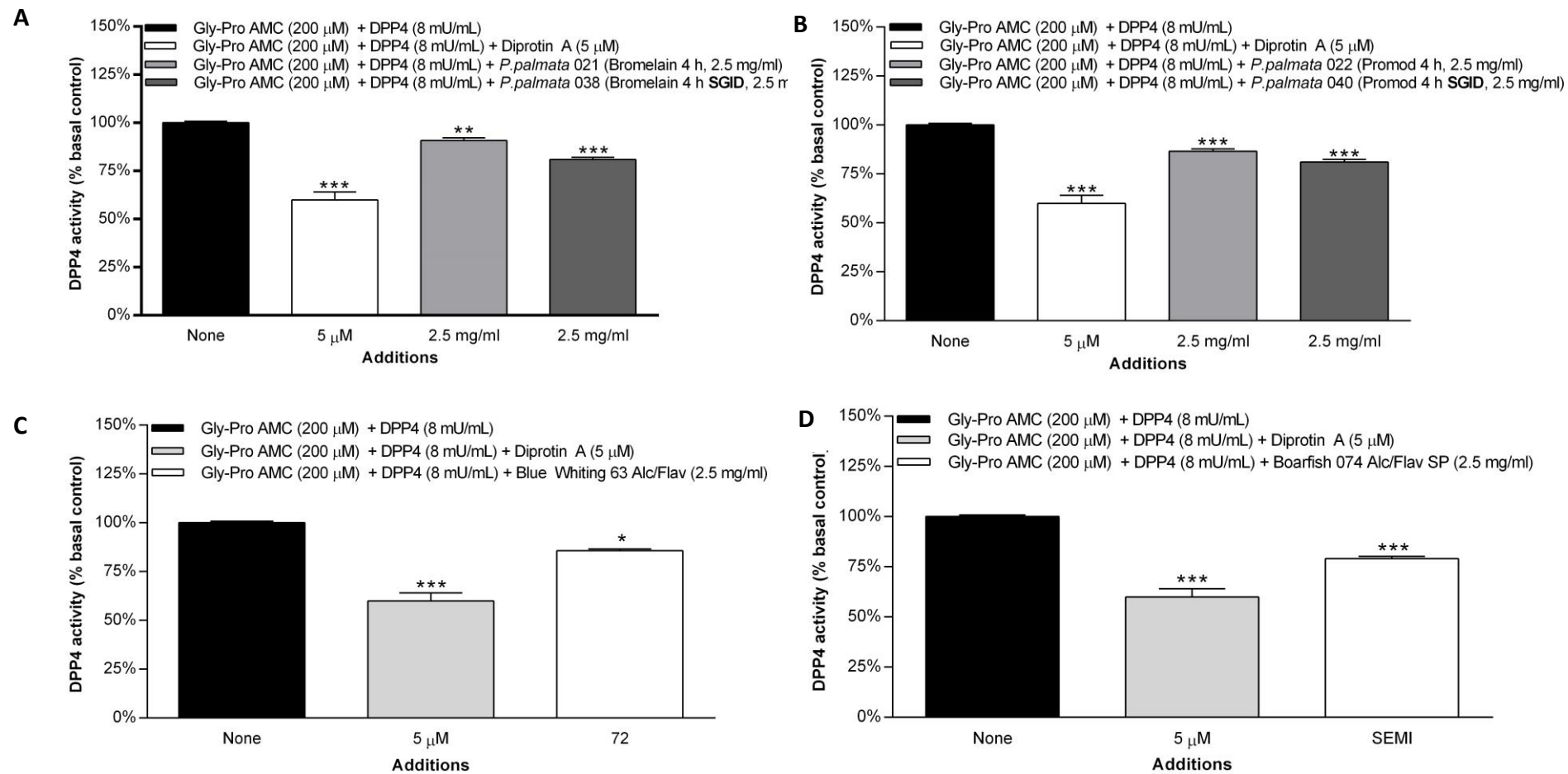


Fig 3.25. Effects of marine protein hydrolysates on DPP4 activity and liberation of the flurogenic AMC substrate. Gly-Pro-AMC (200 μ M) was incubated with DPP4 (8 mU/ml) alone or with either ULPH021 or 038 (**Panel A**), ULPH022 or 040 (**Panel B**) or ULPH063 (**Panel C**) or ULPH074 (**Panel D**) using a fixed concentrations of hydrolysate (2.5 mg/ml) for 30 min with liberated AMC analysed using EX/EM 360/460 nm wavelength. Values are expressed as mean \pm S.E.M. (n=3). *p<0.05, **p<0.01, ***p<0.001 compared to respective DPP4 only control.

3.26

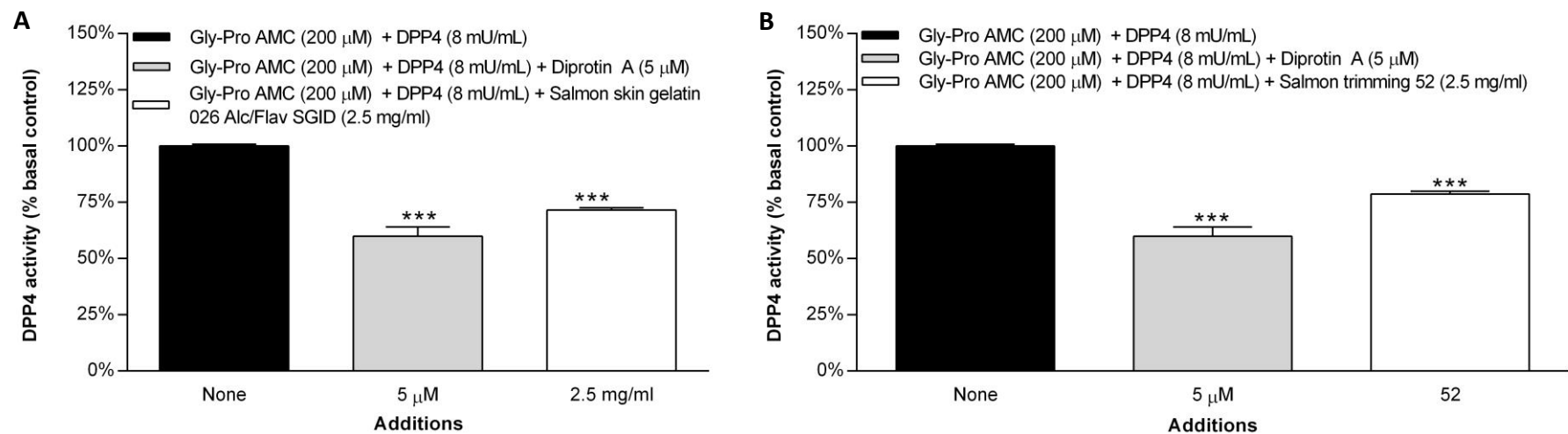


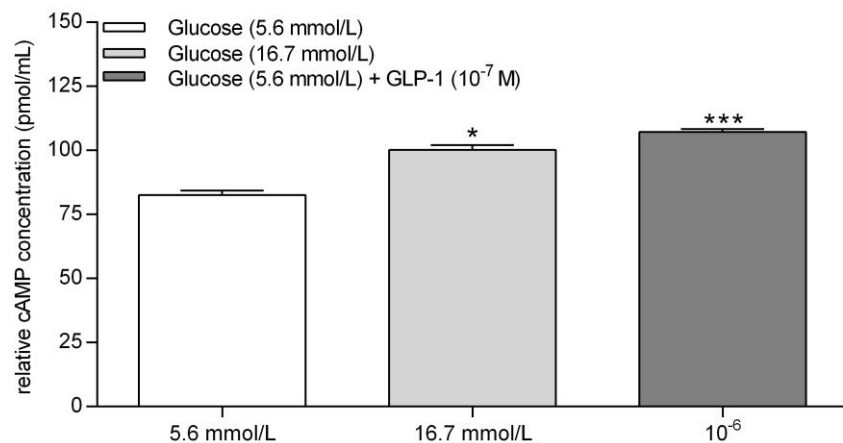
Fig 3.26. Effects of marine protein hydrolysates on DPP4 activity and liberation of the fluorogenic AMC substrate. Gly-Pro-AMC (200 μ M) was incubated with DPP4 (8 mU/ml) alone or with either ULPH026 (**Panel A**), ULPH052 (**Panel B**) using a fixed concentrations of hydrolysate (2.5 mg/ml) for 30 min with liberated AMC analysed using EX/EM 360/460 nm wavelength. Values are expressed as mean \pm S.E.M. (n=3). ***p<0.001 compared to respective DPP4 only control.

Table 5 – DPP-4 activity summary

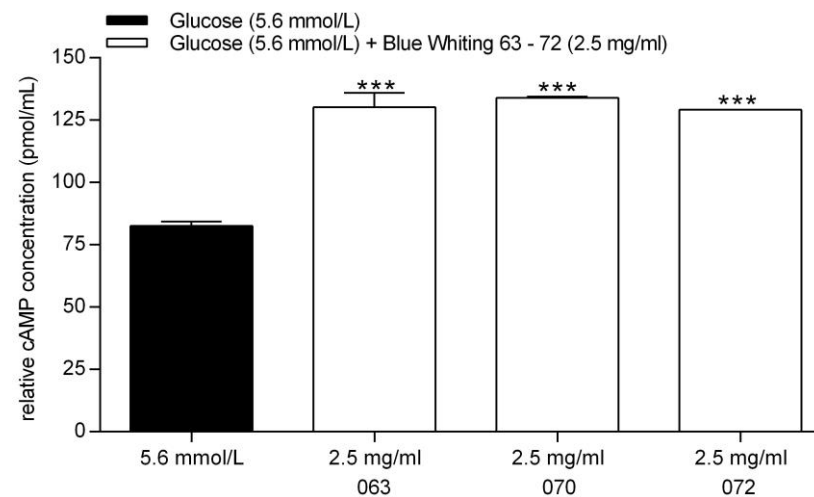
Flurogenic AMC liberation via DPP4 activity summary					
Hydrolysate	Source	DPP4/Hydrolysate	% DPP4 activity decrease	Significance	SGID
Control	Diprotin A	8mU / 0 mg/ml	40%	***p<0.001	
18	<i>P.palmata</i>	8mU / 2.5 mg/ml	32%	***p<0.001	n
42	<i>P.palmata</i>	8mU / 2.5 mg/ml	30%	*p<0.005	y
19	<i>P.palmata</i>	8mU / 2.5 mg/ml	24%	***p<0.001	n
34	<i>P.palmata</i>	8mU / 2.5 mg/ml	17%	*p<0.005	y
20	<i>P.palmata</i>	8mU / 2.5 mg/ml	35%	***p<0.001	n
36	<i>P.palmata</i>	8mU / 2.5 mg/ml	17%	***p<0.001	y
21	<i>P.palmata</i>	8mU / 2.5 mg/ml	10%	**p<0.01	n
38	<i>P.palmata</i>	8mU / 2.5 mg/ml	20%	***p<0.001	y
22	<i>P.palmata</i>	8mU / 2.5 mg/ml	14%	***p<0.001	n
40	<i>P.palmata</i>	8mU / 2.5 mg/ml	19%	***p<0.001	y
63	Blue whiting	8mU / 2.5 mg/ml	15%	*p<0.005	n
62	Boarfish	8mU / 2.5 mg/ml	15%	**p<0.01	n
74	Boarfish	8mU / 2.5 mg/ml	21%	***p<0.001	n
55	Salmon Trimming	8mU / 2.5 mg/ml	22%	***p<0.001	n
26	Salmon gelatine	8mU / 2.5 mg/ml	29%	***p<0.001	y

3.27

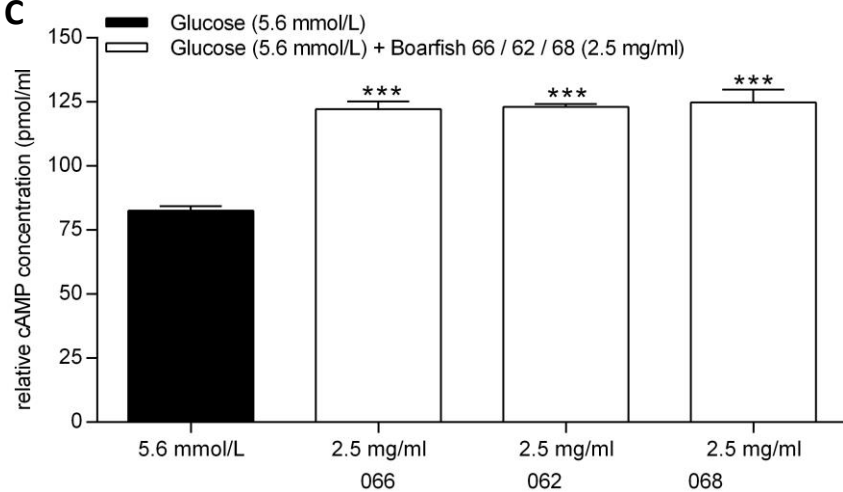
A



B



C



D

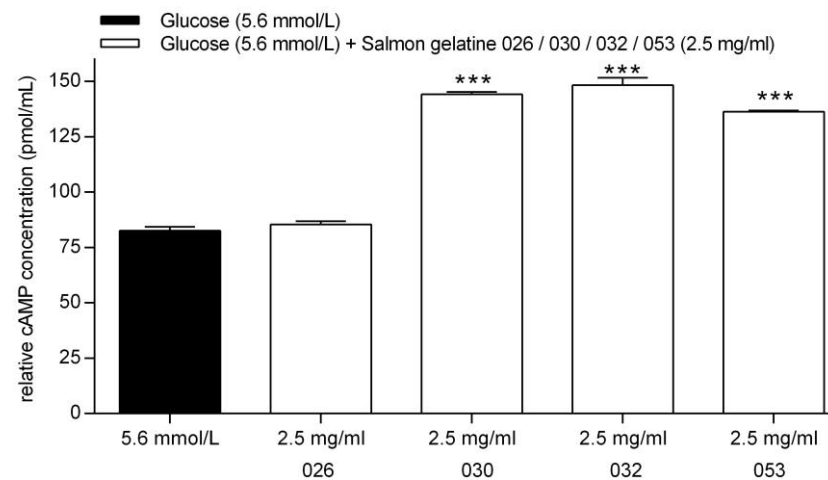
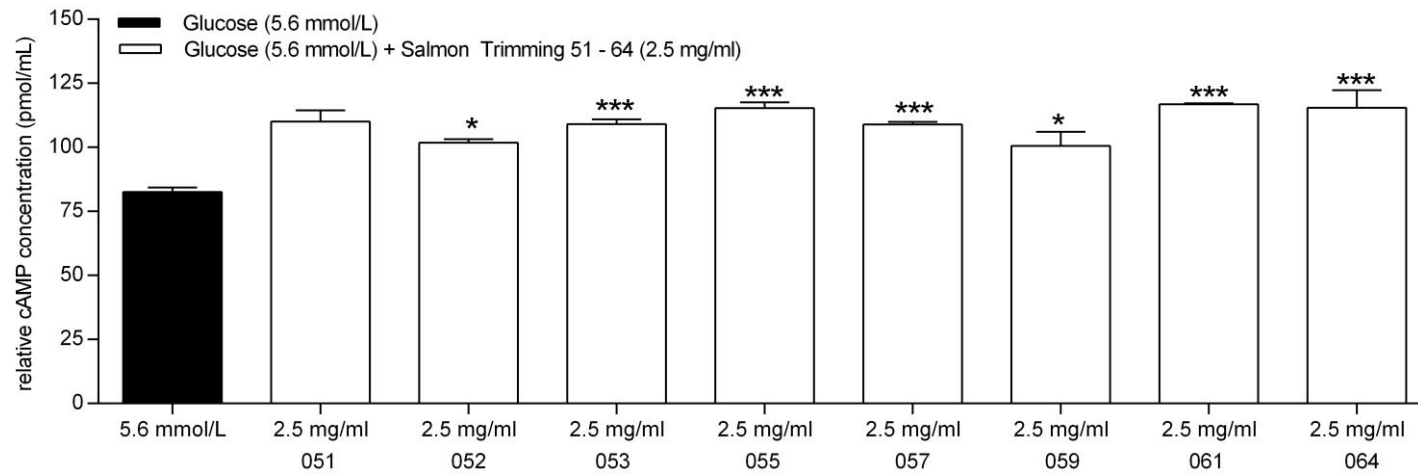


Fig 3.4.27. Effects of marine protein hydrolysates on cAMP production in BRIN-BD11 cells during acute co-incubation with glucose alone or with glucose (16.7 mM) or GLP-1 (10^{-7}) (**Panel A**) or ULPH063 070 or 072 (**Panel B**), ULPH066, 062 or 068 (**Panel C**) or ULPH026, 030, 032 or 053 (**Panel D**) using a fixed concentrations of hydrolysate (2.5 mg/ml) for 20 min. Cells were lysed using cAMP assay lysis buffer with cAMP concentration established using a cAMP specific ELISA. Values are expressed as mean \pm S.E.M. (n=3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to respective glucose only control.

3.28 A



B

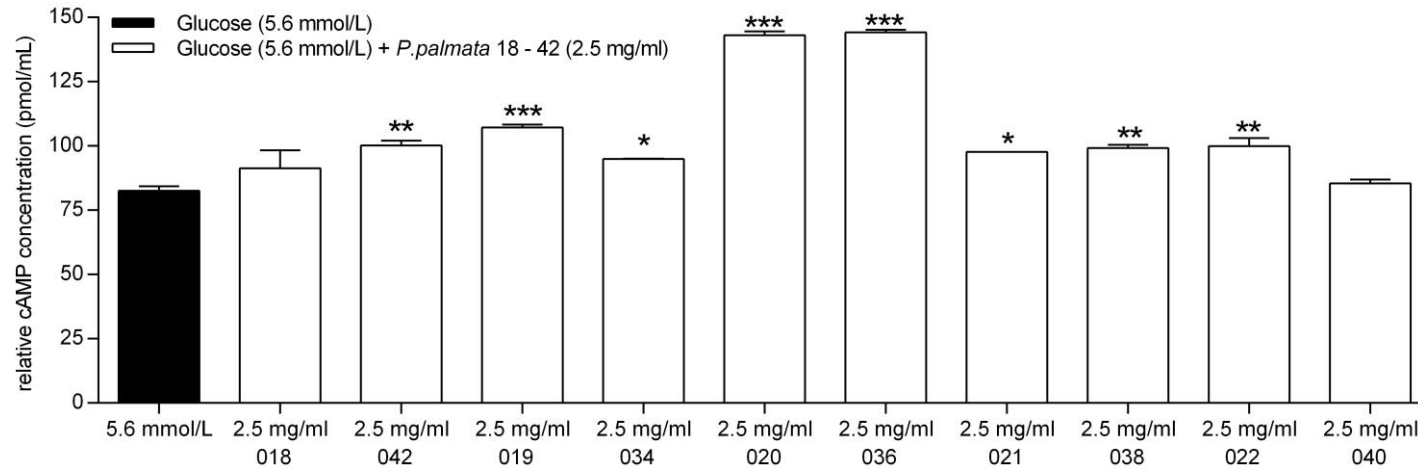


Fig 3.28. Effects of marine protein hydrolysates on cAMP production in BRIN-BD11 cells during acute co-incubation with glucose alone or with ULPH051, 052, 053, 055, 057, 059, 061, or 064 (**Panel A**) or ULPH018, 042, 019, 034, 020, 036, 021, 038, 022, 040 (**Panel B**) using a fixed concentrations of hydrolysate (2.5 mg/ml) for 20 min. Cells were lysed using cAMP assay lysis buffer with cAMP concentration established using a cAMP specific ELISA. Values are expressed as mean \pm S.E.M. (n=3). *p<0.05, **p<0.01, ***p<0.001 compared to respective glucose only control.

3.29

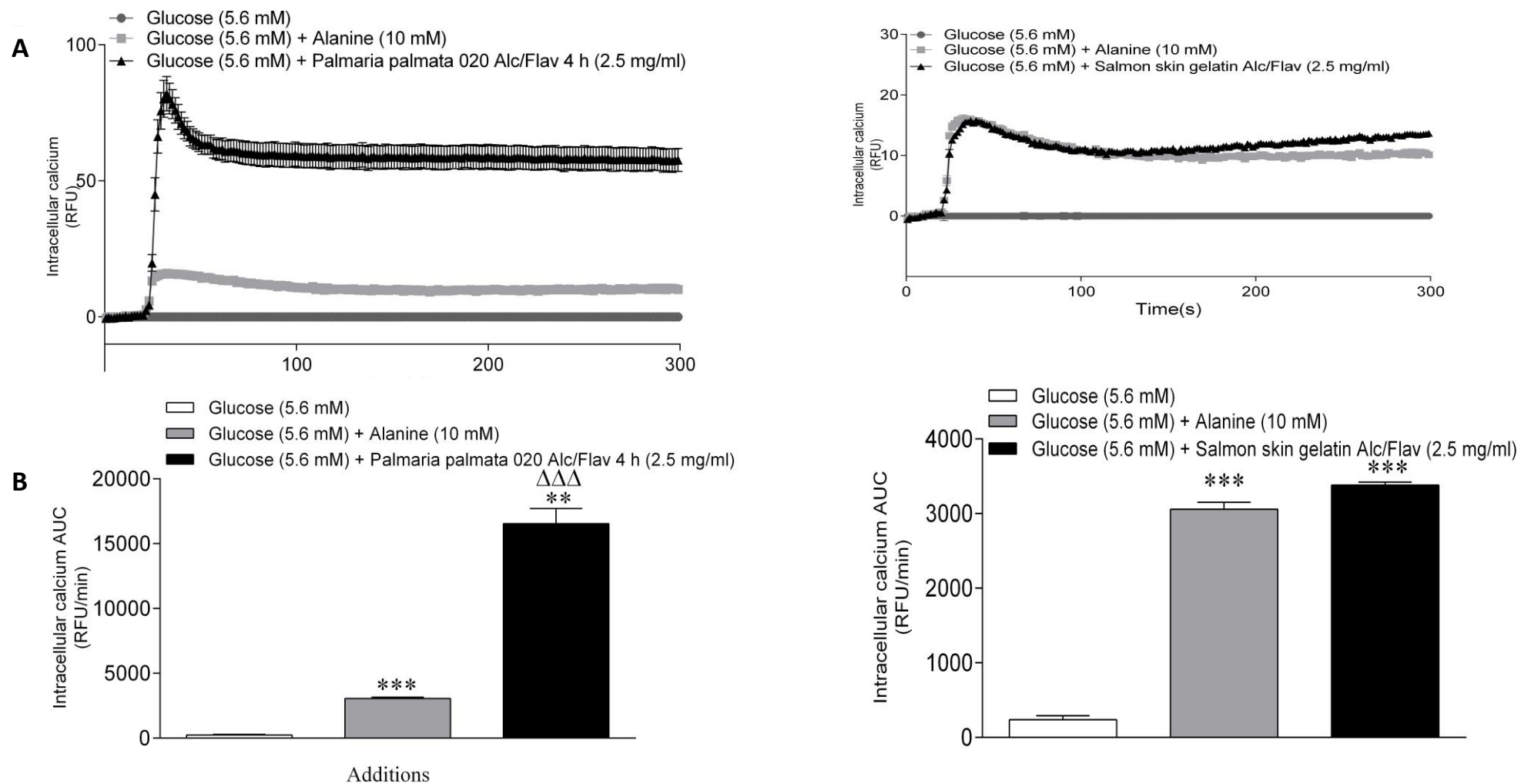


Fig 3.29. Effects of marine protein hydrolysates on intracellular calcium mobilization in BRIN-BD11 cells during acute co-incubation with glucose alone or alanine (10 Mm) or ULPH055 (**Panel A**) or ULPH020 (**Panel B**) using a fixed concentrations of hydrolysate (2.5 mg/ml) for 300 sec. Cells were incubated prior with a proprietary florescent Ca²⁺ and read using a spectrophotometer. Values are expressed as mean \pm S.E.M. (n=3). ***p<0.001 compared to respective glucose only control.

3.30

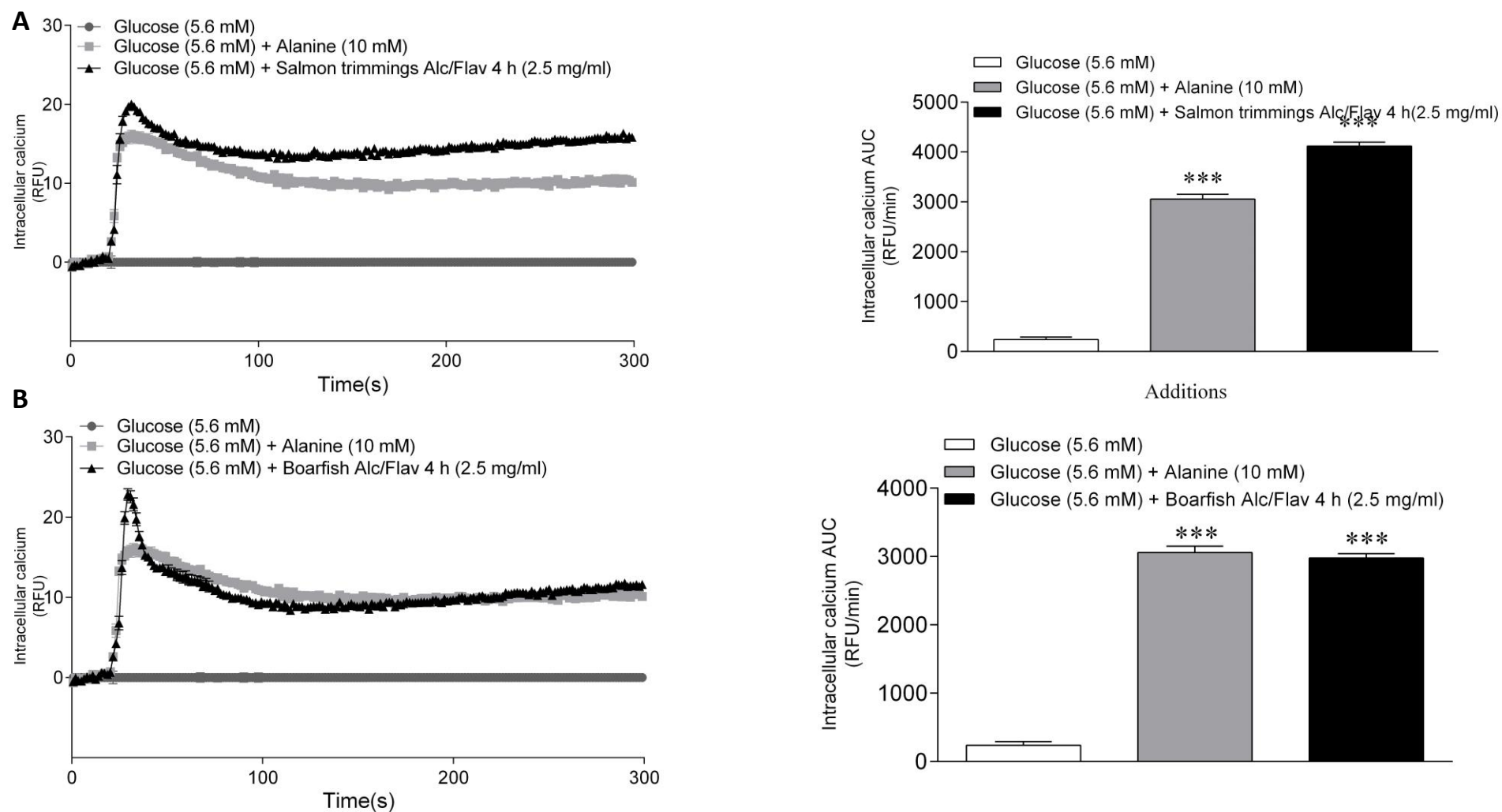


Fig 3.30. Effects of marine protein hydrolysates on intracellular calcium mobilization in BRIN-BD11 cells during acute co-incubation with glucose alone or alanine (10 mM) or ULPH055 (**Panel A**) or ULPH066 (**Panel B**) using a fixed concentrations of hydrolysate (2.5 mg/ml) for 300 sec. Cells were incubated prior with a proprietary fluorescent Ca²⁺ and read using a spectrophotometer. Values are expressed as mean \pm S.E.M. (n=3). Values are expressed as AUC adjacent. ***p<0.001 compared to respective glucose only control.

3.31

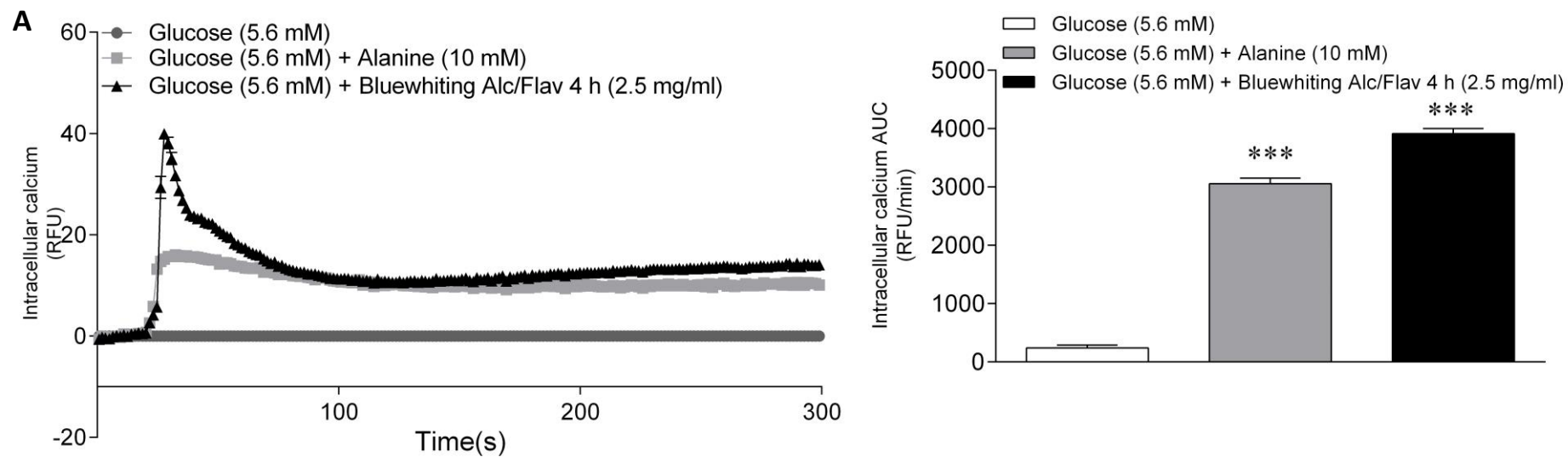


Fig 3.31. Effects of marine protein hydrolysates on intracellular calcium mobilization in BRIN-BD11 cells during acute co-incubation with glucose alone or alanine (10 Mm) or ULPH063, using a fixed concentrations of hydrolysate (2.5 mg/ml) for 300 sec. Cells were incubated prior with a proprietary florescent Ca²⁺ and read using a spectrophotometer. Values are expressed as mean \pm S.E.M. (n=3). Values are expressed as AUC adjacent. ***p<0.001 compared to respective glucose only control.

3.32

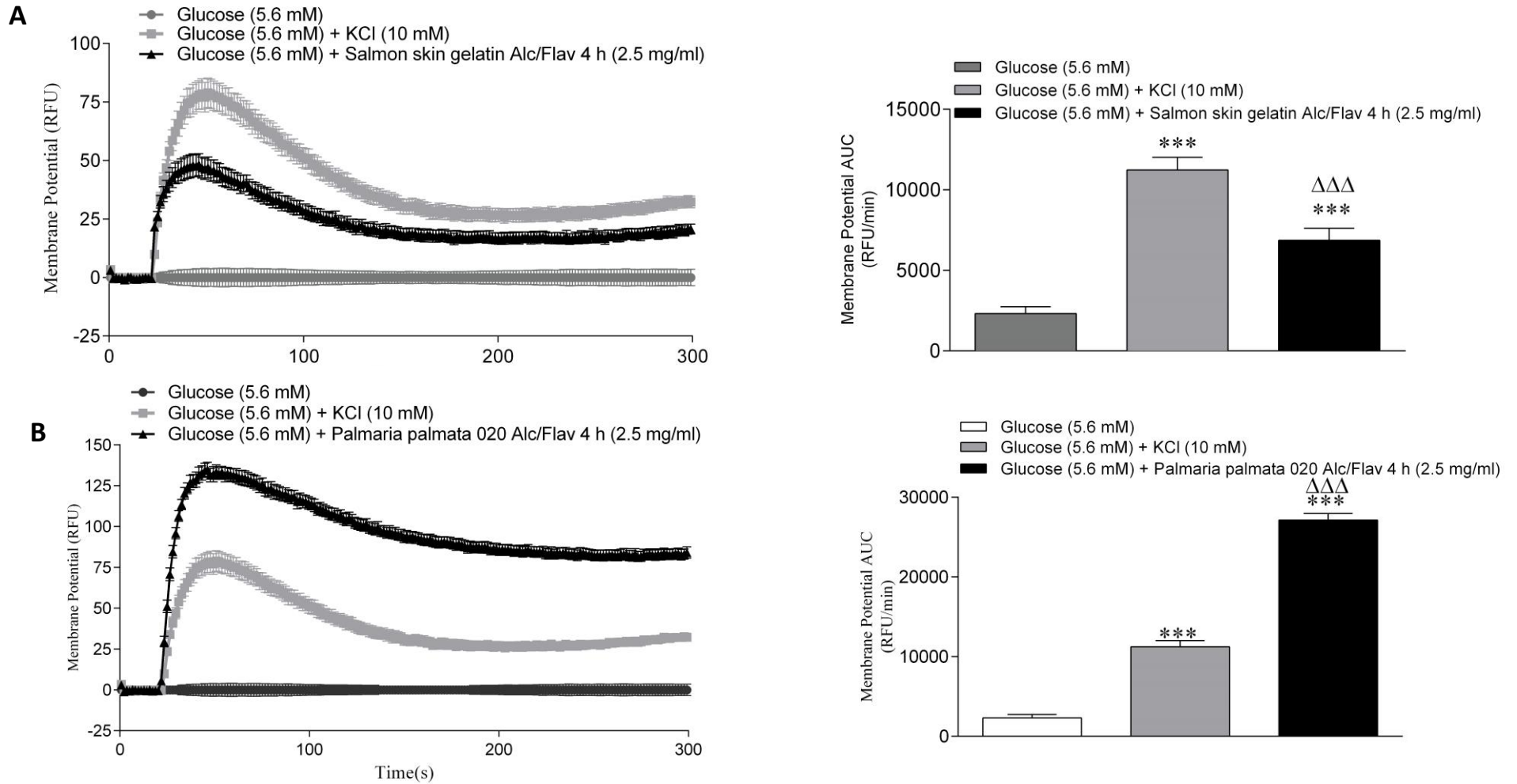


Fig 3.32. Effects of marine protein hydrolysates on membrane potential in BRIN-BD11 cells during acute co-incubation with glucose alone or KCL (10 Mm) or ULPH055 (**Panel A**) or ULPH020 (**Panel B**) using a fixed concentrations of hydrolysate (2.5 mg/ml) for 300 sec. Cells were incubated prior with a proprietary florescent membrane potentiating electrolyte and read using a spectrophotometer. Values are expressed as mean \pm S.E.M. (n=3). ***p<0.001 compared to respective glucose only control.

3.33

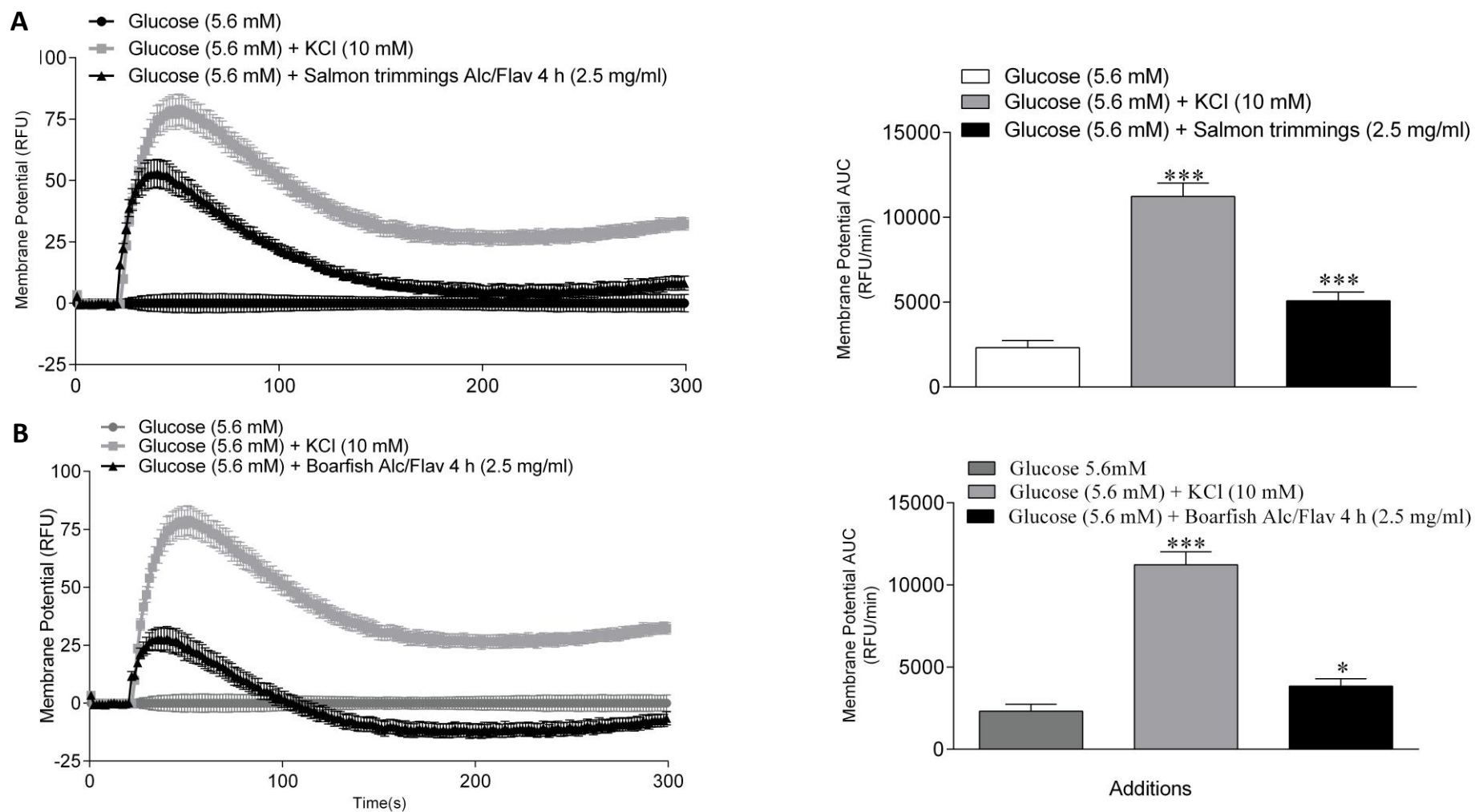


Fig 3.33. Effects of marine protein hydrolysates on membrane potential in BRIN-BD11 cells during acute co-incubation with glucose alone or KCL (10 Mm) or ULPH055 (**Panel A**) or ULPH062 (**Panel B**) using a fixed concentrations of hydrolysate (2.5 mg/ml) for 300 sec. Cells were incubated prior with a proprietary florescent membrane potentiating electrolyte and read using a spectrophotometer. Values are expressed as mean \pm S.E.M. (n=3). * p <0.05, *** p <0.001 compared to respective glucose only control.

3.34

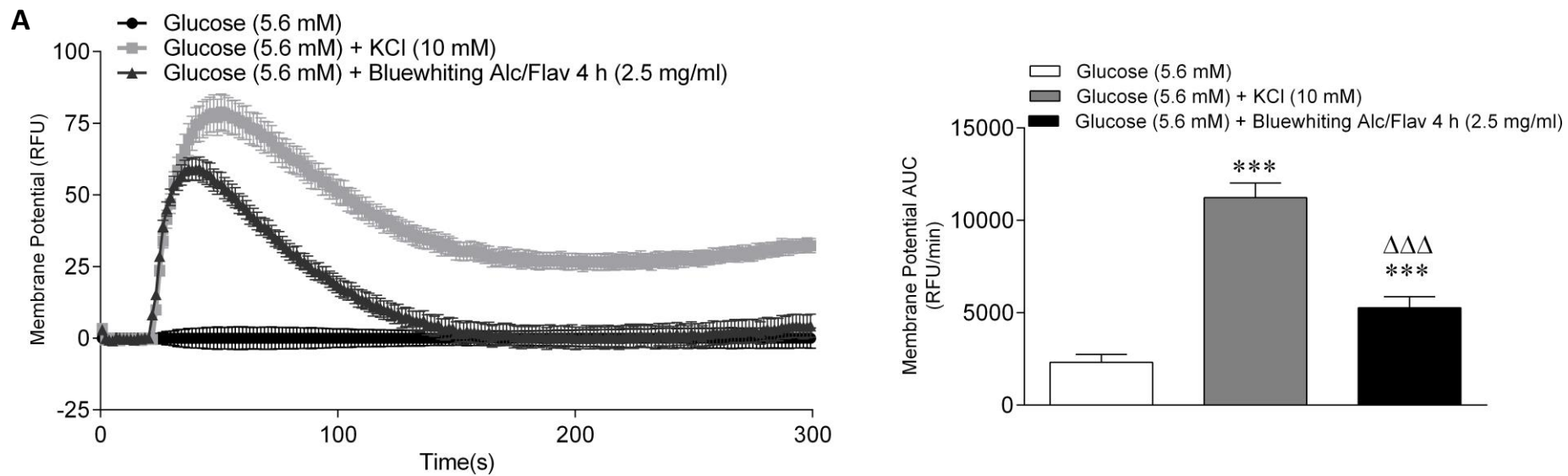


Fig 3.34. Effects of marine protein hydrolysates on membrane potential in BRIN-BD11 cells during acute co-incubation with glucose alone or KCL (10 Mm) or ULPH063 (**Panel A**) using a fixed concentrations of hydrolysate (2.5 mg/ml) for 300 sec. Cells were incubated prior with a proprietary florescent membrane potentiating electrolyte and read using a spectrophotometer. Values are expressed as mean \pm S.E.M. (n=3). ***p<0.001 compared to respective glucose only control.

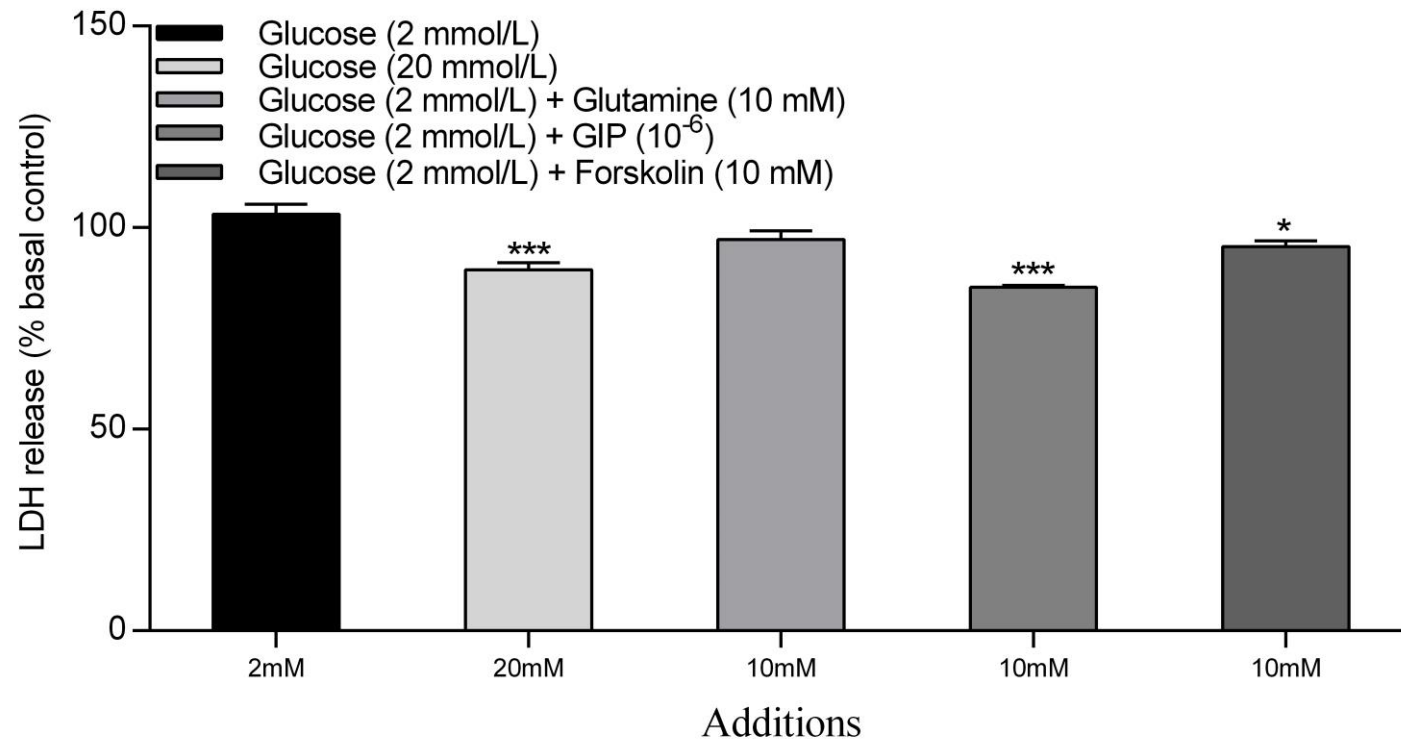
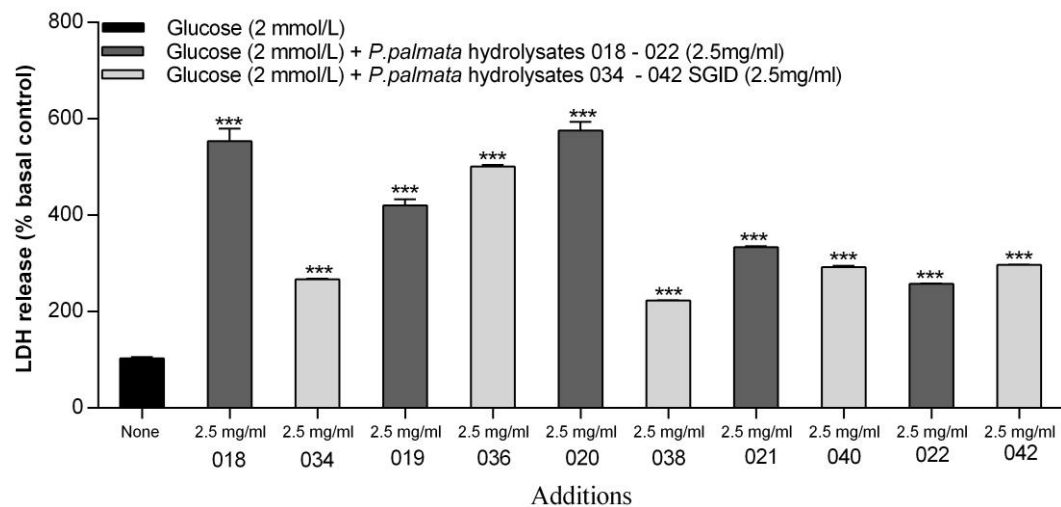


Fig 3.35. Effects of secretagogues on cellular toxicity and LDH release in cells during acute co-incubation with glucose alone or glucose (20 mM), Glutamine (10 mM), GIP (10^{-6}) or Forskolin (10 mM) (**Panel A**). Cells were incubated acutely (20 minutes) with test conditions with supernatant tested for LDH release from cultured cells during experimental investigation. Values are expressed as mean \pm S.E.M. (n=3). *p<0.05, ***p<0.001 compared to respective glucose only control.

A



B

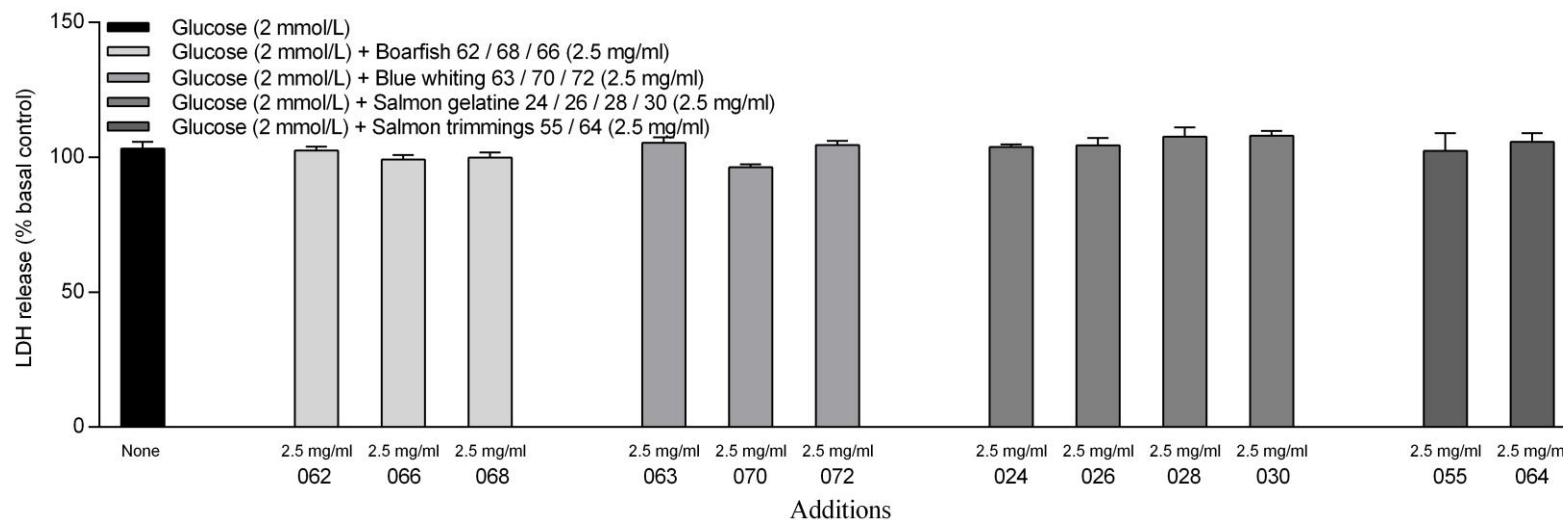


Fig 3.36. Effects of marine protein hydrolysates on cellular toxicity and LDH release in cells during acute co-incubation with glucose alone or ULPH018, 034, 019, 036, 020, 038, 021, 040, 022 or 042 (Panel B). Cells were incubated acutely (20 minutes) with test conditions with subsequent supernatant tested for LDH release from cultured cells during experimental investigation. Values are expressed as mean \pm S.E.M. (n=3). ***p<0.001 compared to respective glucose only control.

3.37

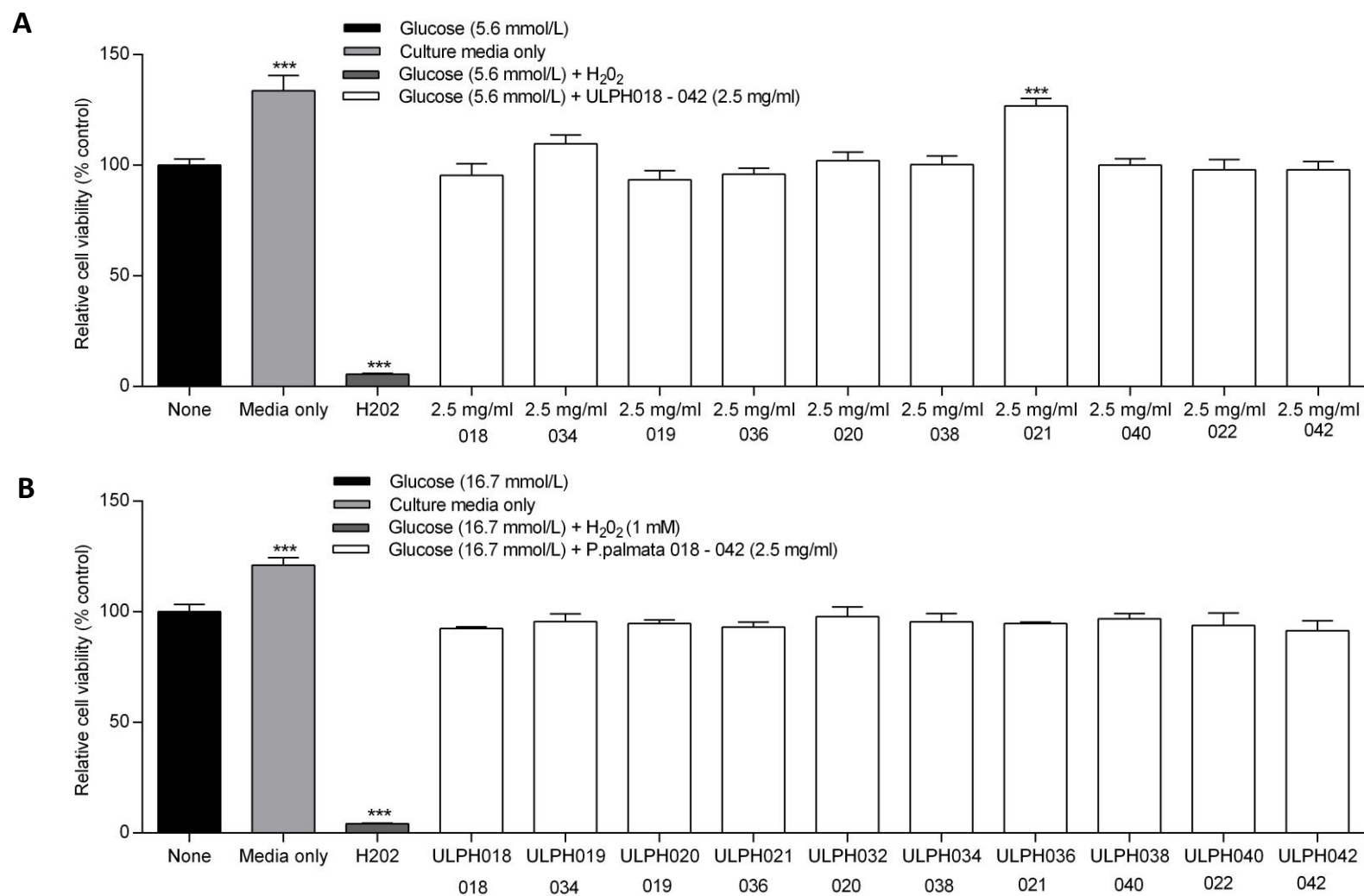


Fig 3.37. Effects of marine protein hydrolysates on cellular viability and respiration BRIN-BD11 cells at 5.6 mmol/L (**Panel A**) and 16.7 mmol/L glucose (**Panel B**) during acute co-incubation with glucose , alone or glucose and ULPH018, 034, 019, 036, 020, 038, 021, 040, 022, 042 (Panel A/B). Cells were incubated acutely (20 minutes) with test conditions of which were aspirated and replaced with growth media containing MTT for 2 hours. Values are expressed as mean \pm S.E.M. (n=3). ***p<0.001, Values are compared to respective glucose only control.

3.38

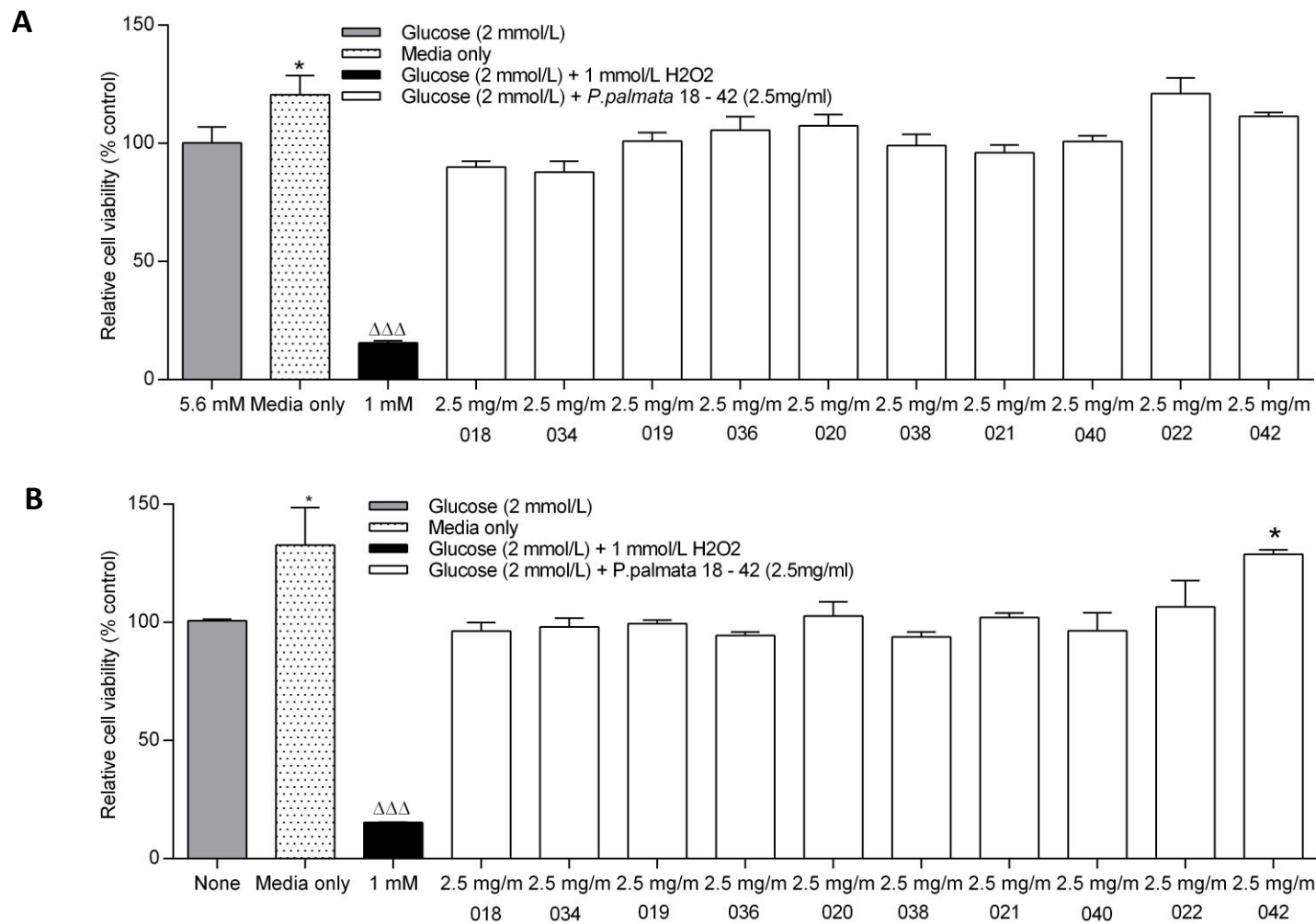


Fig 3.38. Effects of marine protein hydrolysates on cellular viability and respiration GLUTag cells at 2 mmol/L (**Panel A**) and STC-1 cells at 2 mmol/L glucose (**Panel B**) during acute co-incubation with glucose alone or glucose and ULPH018, 034, 019, 036, 020, 038, 021, 040, 022, 042. Cells were incubated acutely (20 minutes) with test conditions of which were aspirated and replaced with growth media containing MTT for 2 hours. Values are expressed as mean \pm S.E.M. (n=3). *p<0.05, ***p<0.001, Values are compared to respective glucose only control.

3.4 Discussion

Initially a cell based approach was used to investigate bioactivity of crude protein hydrolysates generated from marine sources using cell specifically tailored to glycaemic regulation that may have potential to be a target for therapeutic benefit. The role of dietary proteins has in the past been focused primarily on caloric need rather than additive to health, however several decades of bioactive food derived peptide investigation has become increasingly acknowledged (Nasri, 2017). Proteins of interest are becoming ever novel, typically dairy or plant sourced that have undergone aqueous hydrolysis were the highpoint of bioactive peptide research, however new approaches are developed aiming to optimise the hydrolysis processes and characterise the biomolecules generated. This approach has yielded further bioactivity but also enhanced development of collaborations within industry and research communities (Hernández-Ledesma et al, 2014).

Understanding the benefits of the hydrolysis process has improved targeted outcomes of crude protein hydrolysates. This has advanced recently due to the ever expanding list of proteases and peptidases that have specific amino acid interactions and if used correctly has the ability to yield new and exciting peptide molecules of which may be screened for health promoting effects (Shimizu & DO, 2013). To date food derived peptides have been shown to exert various activities on the immune system, cardiovascular and nervous system along with improving blood pressure and digestive health (Daliri et al, 2017). The technologies surrounding the identification of novel bioactive peptides has progressed and become increasingly optimised. The application of peptides for health and in particular for improving insulin secretion via incretin hormone secretion, direct insulin secretion via pancreatic interaction or the activation of satiety centres is not uncommon. Peptide-based treatment approaches is leading the revolution in highly specific diabetes therapeutics due to their multifunctional properties.

Within this chapter, in order to evaluate if marine protein hydrolysates presented bioactivity at the cellular level we employed several secretory cell lines tailored towards diabetes biomarkers. Initially an insulin secreting cell line, BRIN-BD11 was used to investigate the insulinotropic activity of the small molecular weight peptides. Pancreatic β -cell secretion of insulin responds well to nutrient stimuli, specifically glucose, amino acids and free fatty acids (Torres et al, 2009). New approaches acknowledge that peptide hormone-based insulin

secretion may be the most potent approach to improving insulin secretion in those with pancreatic dysfunction. Peptide cellular receptors, specifically the GLP-1r is known to be glucose dependent, and a safe approach to lowering elevated glucose levels via optimised insulin secretion (Meloni et al, 2013). Here we screened a wide array of crude peptides within cultured beta cells, and for the first time displayed several potent insulinotropic dose-dependent hydrolysates. Furthermore, hydrolysates of particular interest were *p.palamata* and boarfish, of which were biologically active at lower concentrations versus that of other sources of hydrolysates. Oral intake of proteins and their insulinotropic actions are commonly reported to firstly arise from gut activation of incretin hormones GLP-1 and GIP including the anorexogenic hormone PYY (Van der Klaauw, 2012). Gut base cells and nutrient sensing hormones are becoming increasingly acknowledge for its potent effect upon glycaemic regulation and promotion of satiety (Salehi et al, 2012). We tested the cellular activation of both GLP-1 secreting L-cells and GIP secreting K-cells using *in vitro* cultured cells. This is a nutritional look-and-see approach to understand the interaction of small molecules and their post-prandial incretin effect (Marathe et al, 2013). Intestinal cell lines respond well to nutrients, in particular amino acids and free fatty acids.

Interestingly, when incubated with a fixed concentration of hydrolysate, secretion of GLP-1 and GIP was general elevated above basal level, however under the same experimental conditions, incretin hormone secretion was lower across all SGID generated hydrolysates, with exceptions of a few. This was rather surprising, as the hydrolysates firstly generated using enzymatic hydrolysis yielded the most potent incretin effect, whereas the further digested SGID hydrolysates resulted in lowered incretin secretion. This reaffirms that these cells respond well to peptides, and less so to amino acids liberated from the peptides after SGID which is contrary to reported work (Lindgren et al, 2015). We further investigated how orally administered peptides would affect endogenous enzyme activity. Two viable approaches currently used within obesity and diabetes management is that of amylase inhibitors and DPP-4 inhibitors. The former decreased the rate of which glucose molecules are liberated from dietary polysaccharide molecules, whereas the latter inhibits a molecule, which shortens the post-prandial incretin effect of GLP-1 and GIP (DiNicolantonio et al, 2015; Plosker, 2014). Investigation enzyme kinetics using an *in vitro* assay approach highlighted that peptide hydrolysates used within, and potentially in general have little effect is any towards

amylase activity, and more specific molecules, such as acarbose are extremely effective. With this noted, peptide based approaches for inhibiting DPP-4 are very exciting, especially food derived approaches (Harnedy et al, 2015; Lacroix & Li-Chan, 2016). The DPP-4 enzyme destroys both GLP-1 and GIP rendering them inactive via cleavage of the first two N-terminus amino acid chains. Inhibiting the enzyme has many advantages, especially towards improved post-prandial glycaemic parameters, satiety promotion and reduction of gastric emptying. Interestingly, SGID hydrolysates had a lesser effect upon inhibiting the enzyme activity using a fluorescence based assay. The hydrolysates prior to SGID had shown as much as a 25% reduction in enzyme activity, which may prolong the incretin effect if used correctly such as preloading prior to meal intake. This affirmed that the interaction and inhibition of DPP-4 was peptide derived, rather than free amino acid derived which would be more prominent within the SGID hydrolysates.

The explanation for peptide bioactivity and its potential beneficial health effects is based upon the inherent amino acid composition and subsequent sequence (Moughan, 2009). This has led researchers to identify an optimised hydrolysis method that is tailored for the protein source, rather than the protein. However, many of these beneficial health effects are contributed from predesigned peptide interactions of which has evolved over millennia. With this being said, targeted peptide discovery from novel sources is an endeavour that has surprising payoffs from overall low drug developmental costs (Zhang et al, 2005).

Investigating bioactive peptides has changed course from larger molecules to small molecular weight molecules ranging from 2 to 20 amino acids in length. Smaller peptide fragments have been shown to exert multifunctional properties and improved intestinal absorption with dietary sources of these being the most important source available (Catnach et al, 1994). In short, we are learning not only that large molecules such as proteins can yield smaller molecules which exert beneficial health effects. In order to assess the bioactivity of protein hydrolysates cellular based signalling pathways were investigated. This involved membrane depolarization, calcium mobilization and generation of cAMP which together allow a general overview of activity to be displayed without delving deeper into specific receptor activation or expression. The hydrolysates were potent towards membrane depolarisation and internal calcium mobilization suggesting cell based activity, however in order to assess if this was potentially via unwanted compounds used within the hydrolysis

process such as electrolytes used for pH ranges, particular focus was spent investigating cAMP. The adenylyl cyclase pathway suggests that small molecular weight peptides may be activating secretion of the aforementioned hormones such as insulin, GLP-1 and GIP via G-coupled protein receptors. These receptors are only activated by extracellular stimuli and can have potent cellular activation via promoting of internal signalling cascades. For example, activation of the GLP-1r via GLP-1 promotes a significant increase in cAMP production and glucose dependent insulin secretion (Ramos et al, 2008). Generally, cAMP was increased across several of the hydrolysates with the exception of salmon trimmings and gelatine. Interestingly, the *p. palmata* aqueous hydrolysate had shown no increase of cAMP, whereas fractions 20 and 36 presented an almost 80% increase during acute co-incubation in BRIN-BD11 cells. This would suggest that within the crude hydrolysates, small molecular weight peptides are activating peptide based GPCRs and furthermore may have further pleiotropic effects that are not solely confined within the pancreas. Lastly, cellular integrity and viability was not effected during acute incubation with each of the hydrolysates. One thing to note however was the misleading results from the LDH assay carried out after incubation with *p.palmata* based hydrolysates. Unfortunately the chlorophyll pigment interfered with the absorbance values of the assay meaning further investigating using the well-established MTT assay was needed. The results confirmed however that no effect upon cellular respiration and viability was present.

We had hypothesized that activity would rely greatly on the hydrolysis process, length of hydrolysis and which protease was used in the generation of the small molecular weight peptides. Initial investigation using BRIN-BD11 cells was initially investigated for insulinotropic activity during co-incubation with test hydrolysates.

Improved insulinotropic activity seen after enzymatic hydrolysis and/or retained activity after SGID is related to the initial peptide fragment length and amino acid sequence, the observed bioactivity of the parent fragment and subsequent fragments that can be produced by further proteolytic digestion. This had reaffirmed that subsequent additional hydrolysis seen yields further smaller peptides that are reported to have multifunctional activity of which improves overall activity of the biopeptides. During oral digestion, protein undergoes rapid digestion with endogenous proteases produced in the stomach such as pepsin, the pancreas such as trypsin and within the small intestines which produce

carboxypeptidases. The aim is to digest protein molecules into their amino acid mono-macronutrient form for absorption, however peptides from 2 to 5 amino acids have been shown to actively pass the intestinal brush boarder and into circulation of which will be investigated in latter chapters. The beneficial effect of crude peptides that are absorbed within the duodenum, jejunum or ileum is speculative as assuming the peptides arrive or get absorbed intact is at this stage unknown, however using cells that originate in these areas such as GLP-1 secreting GLUTag cells and GIP secreting STC-1 cells allowed researchers to approach the antidiabetic potential of marine protein hydrolysates using a nutritional “look and see” approach while measuring incretin hormone release.

The ability of smaller molecules interacting with exponentially larger ones, such as peptide/protein or protein/protein interaction has been of interest for the past decade. Small molecules that can antagonise or agonise an enzyme, cell receptor or cause cellular signalling are of vital importance and can be biologically potent while remaining cost effective to produce over larger increasingly complex molecules. As mentioned previously the enzyme DPP4, a serine peptidase of which preferentially cleaves dipeptides from the N-terminus of a peptide chain and is one of the only endogenous enzyme with the ability to cleave a proline beside the penultimate amino acid. This has challenged researchers to make this enzyme a pharmaceutical target and one that has been hugely successful in the treatment of type 2 diabetes. Understanding that proteins are potential bioactive peptide precursors makes them particularly attractive for the development of functional foods or further identifying specific inhibitory peptides.

The study identified several signalling pathways of interest, each potentially complimentary to how crude unknown molecules interact at the cellular level. Further questions need to be addressed regarding multifaceted signalling of a potentially potent singular peptide molecule within the crude hydrolysate mixture or if the diverse signalling properties is derived from synergistic mechanisms, meaning peptides work in tandem to achieve a desired effect, this work displayed the latter. During signalling mechanism studies, several hydrolysates from *Palmaria palmata*, boarfish, blue whiting and salmon derivatives caused an increase in the intracellular messenger cAMP including potent membrane potential followed by intracellular calcium mobilization. Each of these assays are suggestive of receptor activation via direct signalling effects or signalling transduction. Extracellular signals are

internalized via the form of secondary messengers, production of hormones and other biological molecules of which can be represented here using increased cAMP production as a basis of general cellular activation. These are used to upregulate transcription and secretory signalling factors that has been displayed in this study via insulin, GLP-1 and GIP secretion that was clearly evident throughout. However, while we assume the secretory activity is derived from extracellular signalling there are potential for false positives or increased bioactivity that has been derived from unwanted mechanisms. An example seen within this study is that of *Palmaria palmata*. The protein source, while it had been assumed it is of high quantity and quality may contain numerous cellular signalling molecules that are contained within the micronutrient and trace element profile of the source. *Palmaria palmata* contains a high concentration of calcium, sodium, potassium and chloride as expected from its coastal origination. In order to control for this further desalting approaches may be deployed in future studies, however until now it is assumed that any additive effect of these unwanted electrolytes and trace elements is minimal.

Palmaria palmata presented a further challenge during initial hydrolysate cellular toxicity screening. High concentration of chlorophyll caused false toxicity during lactate dehydrogenase assay investigation. It was initially assumed that the concentration of hydrolysate used caused adverse effects, however until further investigation using the well-established MTT cellular viability assay reaffirmed the safety of the hydrolysate during acute incubation in all cell lines investigated. No toxicity was present in any of the hydrolysates at the chosen experimental concentration.

Conclusion

Using a rapid high throughput approach to test potentially bioactive hydrolysates has its limitation. This approach fails to provide definitive answers on the potential of proteins and those which serve as bioactive precursors. It also fails to answer which peptides are having the bioactivity displayed and if identifying these peptides is beneficial to the study aim. With this noted, this study has attempted to provide a comprehensive screening model for a disease target including the approach best deployed for generating a marine protein hydrolysate with specific or multifaceted bioactivity. The knowledge surrounding enzymatic

digestion of proteins to yield a product with targeted therapeutic bioactivity is improving. We set out to test the acute *in vitro* effects of marine protein hydrolysates from novel sources using various proteolytic methods. The results are thus far promising with positive insulinotropic and incretin secretion activity including enzyme interaction and signalling mechanisms and cellular toxicity, furthermore identifying several standout hydrolysates of which will be further investigated using acute and chronic *in vivo* assessment. To date, there is a gap in the knowledge for the application of protein hydrolysates in general. This study has attempted to rapidly screen marine protein hydrolysates for potential anti-diabetic potential of which will be the sole focus for the remaining of this work.

Trajectory of work from herein

Using the data generated from *in vitro* experimentation, identify which of the hydrolysis methods provides and promotes the most promising secretory activity of insulin, GLP-1 and GIP via *in vitro* experimentation.

Disseminate which source of protein and the subsequent hydrolysates present the highest level of biological activity at the lowest concentrations between each enzymatic method.

Consider combinational treatments due to some hydrolysates showing greater activity than others in particular cell lines.

Identifying if the biological activity presented within chapter 3 is from the peptides generated from protein hydrolysis via the use of synthetically produced peptides identified from a previously generated hydrolysate (Chapter 4).

Identify the dose required to firstly improve postprandial glucose excursion in non-diabetic mice, including oral administration of hydrolysates on satiety and delayed glucose tolerance (chapter 5).

The effect of the most promising hydrolysates identified on markers of diabetes and obesity in genetic and diabetes induced models of diabetes (chapter 6/7).

Finally, identify if the dose used within mice models is translatable to human study and the hydrolysates effect on short-term glucose tolerance and markers of satiety (Chapter 9).

Chapter 4

Screening multifunctional bioactivities of synthetic peptides
derived from *Palmaria palmata* (Dulse) protein hydrolysate

4.1 Introduction

Obesity and related co-morbidities present challenges that have in recent years spiralled out of control becoming a global epidemic. Treatment costs now affect health care centres across multiple departments, which pose to further stretch healthcare budgets worldwide (Seuring *et al*, 2015). The most common, yet avoidable outcome of chronic obesity is type 2 diabetes mellitus (T2DM), which accounts for 90% of global diabetes prevalence and characterised by obesity, chronic hyperglycaemia and dyslipidaemia (Shaw *et al*, 1999). During obesity related progression of T2DM, pancreatic dysfunction disrupts intrinsic glucose homeostatic mechanisms of which are tightly controlled via peptide hormones, insulin, glucagon and somatostatin from β , α and δ cells within the islet of Langerhans (Zyromski, 2015). Cells which are most adversely altered by chronic over-nutrition are the β -cell which accounts for 90% of total islet cell mass within the endocrine tissue, however represent only 1 - 2 % of total cells within the pancreas, in turn this leads to β -cell dysfunctions and subsequent insulin resistance when the disease is established (Mingyu *et al*, 2014; Gupta *et al*, 2012).

Dietary intake has a profound effect on blood glucose concentrations and disease progression, as such, modern strategies implemented focus on reducing carbohydrates and lipids, however no reduction in protein necessary (Frans, 2014). New evidence suggesting classical dietary modification approaches may be enhanced by incorporation of bioactive food components or foods with multifunctional properties that extend further than purely caloric requirements (Oh & Jun, 2014). These approaches however, aim to highlight novel molecules of which constitute only a micronutrient portion of the food of which it is contained within. Newer approaches unlocking bioactive peptides from food-derived proteins within in the last decade has highlighted the importance of food components that may be exploited for therapeutic gain (Patil *et al*, 2015; Xia *et al*, 2017).

Using proteolytic enzymes which unlock small molecular weight peptides with a targeted range of 2 to 20 amino acids has been underlined as a researching aim with huge nutraceutical potential (Oseguera-Toledo *et al*, 2014). Screening of food derived protein by-products has spanned

across several modern key research areas with research highlighting the anti-cancer (Lefranc *et al*, 2017), hypotensive (Rong *et al*, 2014), and anti-obesity properties (Pan *et al*, 2016). Anti-diabetic properties of small peptide molecules is a new endeavour for their intrinsic interaction with endogenous enzymes, signalling and regulatory pathways of which have the ability to display advantageous health outcomes. Endogenous food proteins are vital for health with their smaller degraded peptide molecules and amino acids full-filling vital metabolic roles improving health prior to being utilized in metabolic or cellular respiratory roles (Jain & Chawrai, 2005).

Its proposed that oral nutrient sensing, hormone secreting cells within the intestinal tract are currently a key target area of diabetes therapy (Sanyal, 2013). Improving the action or increasing the circulating concentration of intestinal hormones both glucagon-like-peptide 1 (GLP-1) secreted from intestinal L-cells and glucose-dependant insulinotropic polypeptide (GIP) secreted from intestinal K-cells have numerous beneficial effects including improved post-prandial glucose excursion, glucose-dependent insulin secretion, reduction of gastric emptying, improvement of glycated haemoglobin (HbA_{1c}) (Kim and Egan, 2008) and effects which are seen beyond the pancreas, such as the more-recently highlighted, potential improvement of cognitive function. Both GLP-1 and GIP are rapidly degraded by the transmembrane cell surface expressed serine protease, dipeptidyl-peptidase 4 (DPP4) also known as CD26. The 101 amino-acid enzyme targets the N-terminus di-peptide from the peptide chain of both GLP-1 and GIP analogues rendering the incretin hormones inactive in regards to glucose homeostasis (Dicker, 2011).

DPP4 is overly expressed in several disease states including inflammatory conditions, cancer, obesity and diabetes (Röhrborn *et al*, 2015). Inhibition of the enzymes has been a therapeutic target since the development of the first small synthetic molecule that interacts and renders the enzyme inactive, sitagliptin. Approval of sitagliptin use was granted on October, 2006 and since, several improvements have been made in both biological activity and duration of action of newer DPP4 enzyme antagonist molecules. The biological half-life of saxagliptin (Onglyza) ranges from 2 – 4 hours,

sitagliptin (Januvia) from 8 – 15 hours, were as more modern enzyme antagonists, linagliptin (trajenta) boasts a 24 hour half-life with the aim of improving patient compliance and improvement of post-prandial glucose regulation. Currently 13 DPP4 inhibitors are in use or awaiting approval via FDA regulatory bodies. The efficacy and safety of DPP4 inhibitors has improved and recently evolved into combinational drug approaches in a single tablet form, such as the sitagliptin/metformin combination (5mg / 500 mg w/w), marketed as Janumet.

The mechanism, specificity and duration of DPP4 inhibitors rely on enzyme interaction, with each having diverse structural protein binding sites with several functional areas across the enzyme promoting decreased kinetic activity. A few of the most potent molecules target the proline mimetic, cyanopyrrolidine P1 group which increases enzyme inhibitory potency over other targeted protein regions (Thornberry & Gallwitz, 2009)

The DPP4 enzyme was first reported as having the ability of being able to cleave a proline or alanine residue from the penultimate N-terminus amino acid, denoting the name, di-peptidyl peptidase, however more recently the interaction of the enzyme to cleave other amino acids has been reported (Matteucci & Giampietro, 2009). With this noted, several studies have reported DPP4 inhibition using animal and plant derived compounds including peptides obtained via enzymatic hydrolysis of proteins from various biological sources (Jao *et al*, 2015; Liu *et al*, 2017; Harnedy *et al*, 2015). Enzymatic hydrolysis of proteins yields abundant small molecular weight peptides of which have reported multifunctional properties, including incretin hormone secretion and intrinsic enzyme interaction with various biological enzymes such as ACE (Iwaniak *et al*, 2014) and DPP4 (Power *et al*, 2014), respectively. Oral intake of specific food compounds for therapeutic health gain is a relatively new approach for the treatment of diabetes, however as DPP4 is expressed highly within the intestine, oral treatment approaches have been the pharmacotherapy target, this in turn has promoted research to identify novel compounds with DPP4 inhibitory action.

Peptides yielded post-hydrolysis are diverse with various molecular weight, structure and amino acid sequence variations that can result in diverse molecular bioactivity that is translated into effects that are, A.) challenging to characterise in their crude state and B.) require increasing quantities of crude product to display bioactivity over an identified synthetic peptides that may have specific therapeutic capabilities. Oral intake of crude peptides are hypothesised to remain bioactive and resistant to digestion while able promote cellular signalling firstly via the gastrointestinal hormone secreting cells and secondly have more specific cellular interaction based upon the assumption that the smaller peptides of interest will be absorbed intestinally, again proving problematic to establish the source of bioactivity (Sable *et al*, 2017).

This leads researchers to verge from crude peptide screening to targeted identification of the individual peptide/s that may promote a therapeutic effect of interest. Peptides are important natural products, which are present in many terrestrial and aquatic species, which have in more recent times displayed nutraceutical and medicinal properties due to a broad spectra of reported bioactivities (Jo *et al*, 2016). With this being noted, certain peptides can fulfil more than a singular biological role, however their primary use is that of a targeted therapeutic effect. In regards to health, peptides have the ability to regulate cellular proliferation (Nekhai *et al*, 2000), inflammation (Ialenti *et al*, 2001), cell migration and wound repair (Yamada, 2000), and targeted drug delivery (Bruno *et al*, 2014). This approach has lead researchers and industry to produce peptide analogues, which has opened a new door to a generation of targeted healthcare. Peptides can be highly specific with over 60 approved by the FDA and a further 200 in clinical trials and now estimated 800+ in pre-clinical trials. Peptides are now recognised as being highly selective, well tolerated and effective, of which is contrived versus current diabetes therapy approaches reporting potential hypoglycaemic, pancreatitis and increased cardiovascular events.

Chapter 3 characterised the acute bioactivity of crude protein hydrolysates from various biological marine sources. These nitrogenous sources were carefully chosen based on source

sustainability, availability, protein content, as well as utilizing various processing waste discard sources. The assumption that each of the hydrolysates investigated in the previous chapter present bioactivity via similar cell signalling, hormone secretory activity and enzyme interaction via the same mechanisms or via a single peptide is considered in this case, inaccurate with effects seen from a wide array of unknown peptides having a multifaceted effect. In order to test this hypothesis, several peptides had been identified from protein obtained from *palmaria palmata* protein hydrolysate generated using the proteolytic enzyme, promod. The peptides were firstly identified via hydrolysate RP-HPLC fractioning, Tandem LCQ/MS-MS de-novo sequencing and finally synthesized. Peptides identified ranged from 4 to 11 amino acids in length of which several have been previously identified as potent DPP4 inhibitors (Harnedy *et al*, 2015b). Three of the most potent DPP4 inhibitor peptides, ILAP (ILE-LEU-ALA-PRO) , LLAP (LEU-LEU-ALA-PRO) and MAGVDHI (MET-ALA-GLY-VAL-ASP-HIS-ILE) , were deployed to investigate signalling activity other than the already reported DPP4 inhibitory action, using a range of cell pancreatic and intestinal cell lines with specific antii-diabetes potential including *in vivo* animal models.

Background & Aims

A major limitation to protein hydrolysate investigation is establishing the bioactive component/s contained within a sample. A common approach used is fractionation of peptide components followed by mass spectrometry and peptide structure/amino acid conformity using proteomic tools. Futhermore this allows peptide synthesis and administration of pure identified peptides using similar experimental protocols. Here, three peptides previously identified from *Palmaria palmata* protein hydroysates, are known to reduce the activity of DPP4, a current pharmaceutical target for the treatment of type 2 diabetes. We aim to further investigate the biological activity of these peptides across multiple cell cultures including the administration to small rodent models alone, or in combination with GLP-1, a hormone

typically cleaved to its inactive form by DPP4 rendering its effect upon glucose excursion redundant. We hypothesize that the identified synthetic peptides will present *in vitro* bioactivity and furthermore improve the effect upon glucose excursion in mice after being injected alone, or in combination with GLP-1 if these peptides possess the ability to inhibit DPP4 as previously shown *in vitro*.

4.2 - Materials and Methods

HPLC elution properties of peptides from crude *Palmaria palmata* protein hydrolysate

Using a fixed dose of 2.5 mg/ml, hydrolysate sample was first reconstituted in dH₂O (1 ml) and briefly vortexed. Prior to RP-HPLC, sample was centrifuged at 3000 rpm for 20 minutes and supernatant finally filtered using a 0.45 µm whatman filter. The HPLC column equipped was a C18 preparative LUNA (250 x 10 mm, 10 µM particle size) with the flowrate set to 5 ml/min. Solvents consisting of (solvent A) 100% deionized HPLC grade water (dH₂O) supplemented with 0.12% TFA increasing over 60 minutes to 70% of (Solvent B) 70% acetonitrile, 30% dH₂O (70/30 v/v) supplemented with 0.1% TFA. For full RP-HPLC protocol refer to section 2.2.9.

Acute insulin secretory activity in BRIN-BD11 cells

In brief, cells are seeded at a cell density of 1.5×10^5 cell/well in 1 ml of RPMI cell culture media and left for 21 hours to adhere. Insulin radioimmunoassay (RIA) was used for the determination of insulin from both insulin secreting cell lines and from ex-vivo biological plasma samples (Flatt & Bailey, 1981), described in 2.1.3.

GLP-1 secretion from GLUTag mouse enteroendocrine cells

On experimentation day DMEM culture media is removed, cell prepping KRBB (1 mmol/L glucose) is added for 2 hours then removed and further supplemented with KRBB of which contains 2 mmol/L glucose in all conditions (baseline) with compounds of interest used in a dose dependant manner in n=4 wells minimum. A detailed outline is described in 2.2.2 including ELISA protocol in section 2.2.4.

GIP secretion from STC-1 mouse enteroendocrine cells

As outlined in section 2.2.3, cells were grown until 80–90 % confluence. Cells were trypsinized and seeded onto 24 well plates at a seeding density of 15×10^5 for 24 hours. A detailed outline is described in 2.2.3 including ELISA outline in section 2.2.5.

Cellular toxicity using LDH cytotox assay

Experimentation was carried out exactly as described in 2.1.2 with a final step of testing 50 μ l of collected supernatant aliquoted into 96 well plates using test conditions (N=3) for released LDH concentration. Refer to section 2.2.16 for full experimental protocol.

Acute Intracellular cAMP production

BRIN-BD11 cells were cultured as described in section 2.1.3. Cells were seeded on 24 well plates as described in 2.2.1. Cells were then lysed after removal of supernatant using 100 μ l of cell lysis buffer for 30 minutes at 37°C. Cells were visually inspected to verify cell lysis using a phase contrast microscope (Zeiss, Germany). Samples were stored at -20°C until analysis. For an in-depth protocol including the cAMP ELISA used see section 2.2.10.

Investigating GLP-1 activity in the presence of DPP4 using RP-HPLC chromatography

Preserving GLP-1 (7-36_{amide}) from degradation to GLP-1 (9-36) in the presence of DPP4 was achieved by co-incubating with synthetic peptides of marine origin first isolated from *Palmaria palamta*. A 100mM Triethanolamine (TEA) buffer (pH 7.4) was used as a biological

buffer of which all reagents would be dissolved in. Protocol in brief, 30 µl of GLP-1^{7-36amide} (10⁶ M) is added to a low bind Eppendorf tube (Sigma-Aldrich, Dorset, UK) followed by 30 µl of sample, control (10⁶ M) or blank. Following this 430 µl of TEA buffer is added. The reaction is started by adding 10 µl of DPP4 (5 mU). Several tubes containing the same additions are prepared and subsequently incubated for 0 h, 2 h, 8 h and 24 h respectively. The reaction is stopped by adding 50 µl of 10% TFA. Samples are then stored at -20°C until RP-HPLC analysis. Refer to section 2.2.9 for HPLC and solvent protocol information.

Compound mass^{da} determination using Matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS)

For full MALDI-TOF MS experimental protocol, refer to section 2.2.10.

Acute glucose lowering effect of peptides via intraperitoneal injection (IPGTT)

The animal model used were NIH Swiss mice (section 2.3.1). Each group (n=8) were fasted for 8 hour prior to experimentation and housed as described in 2.3.1. Food was withheld during experimentation however access to water was available. Prior to compound administration a fasting blood sample was analysed for glucose concentration using a handheld glucometer (Bayer Contour, Leverkusen, Germany). A fixed dose (25 nmol/kg bodyweight) of peptide was then mixed with a dose of glucose (18.8 mmol/kg bodyweight) and administered via IP injection. Effect of the peptides was then established by further testing blood glucose concentration at various timepoints over 2 hours (15, 30, 60, 90 and 120 min). After

experimentation was complete animals were given access to food and given a 72 hour washout period before experimentation could be carried out on the same group of animals.

Investigating reduction of food intake in 3 hour trained feeding HsdOla:TO mice

Hsd:Ola:TO mice (8 weeks old) were obtained from Envigo, Blackthorn, UK. For housing and animal conditioning please refer to section 2.3.4. Before animals were given access to food for 3 hours, Food averaging 10 g was weighed. Animals (n=8) were given a chosen compound within a saline vehicle (0.9% NaCl) with food given and weight subsequently every 30 minutes for 3 hours total. Food is then be removed until the following day.

Statistical analysis

Data was expressed as mean \pm SEM with values compared using one-way analysis of variance (ANOVA) followed by post-hoc students T-test. Data between groups were considered statistically significant if $p \leq 0.05$. Data was plotted and analysed using Graphpad Prism 5.

4.3 Results

Fig 4.1 - RP-HPLC separation of crude peptides after enzymatic digestion.

Using the method described in 4.2.1, 4 mg/ml of crude hydrolysate was injected with subsequent peaks analysed over a 60 minute solvent gradient elution period. The chromatograph (Fig. 4.1) displayed was not used in the generation of the peptides identified of which gives an understanding of the hydrophobicity profile of peptides generated using the Alcalase/flavouzyme enzyme combination with protein from *palmaria palmata*. (Fig. 4.1)

Fig 4.2 - Insulinotropic activity of peptides during acute (20 min) co-incubation in BRIN-BD11 cells

Insulin secretion was determined over a 20-minute co-incubation period with each peptide incubated in the presence of a fixed glucose concentration (5.6 mmol/L). Baseline insulin secretion was established using KRBB buffer alone containing 5.6 mmol/L glucose (Fig 4.1 A). All other test conditions contained addition peptide compounds or known insulinotropic agents. The positive controls used were KCL (30 mM) and GLP-1 (10^{-6}) displayed 4.2 –fold ($p < 0.001$) and 3.2 -fold ($p < 0.001$) insulin secretion versus baseline (5.6 mmol/L) glucose, respectively. Peptide LLAP and ILAP displayed a 2 – fold increase in insulin secretion at 10^{-6} M ($p < 0.001$; Fig 4.2 B) and a 1.5 – fold activity in a dose dependant manner to 10^{-9} M and 10^{-11} M ($p < 0.01$ Fig 4.2 C).. Under the same incubation conditions, peptide MAGVDHI (Fig 4.2 D) failed to promote insulin secretion beyond the basal glucose control, respectively.

Fig 4.3 A - Glucagon-like-peptide-1 (GLP-1) secretion of peptides during acute (2 H) co-incubation in enteroendocrine GLUTag cells

GLP-1 secretion was determined over a 2 H co-incubation period with each peptide incubated in the presence of a fixed glucose concentration (2 mmol/L). Basal GLP-1 secretion was established using KRBB buffer alone containing 2 mmol/L glucose (Fig 4.3 A). All other test conditions contained addition peptide compounds or known GLP-1 secreting agents. The positive control used was forskolin (10 mM) which displayed 10 -fold GLP-1 concentration versus the basal glucose control, respectively. Under the same incubation conditions, peptide ILAP (10^{-6} M;), LLAP (10^{-6} M) and MAGVDHI (10^{-6} M) failed to promote any GLP-1 secretion above the basal control from the concentrations used, respectively. (Fig 4.3 A).

Fig 4.3 B - Glucose-dependent insulinotropic-polypeptide (GIP) secretion of peptides during acute (2 H) co-incubation in enteroendocrine STC-1 cells

GIP secretion was determined over a 2 H co-incubation period with each peptide incubated in the presence of a fixed glucose concentration (2 mmol/L). Basal GIP secretion was established using KRBB buffer alone containing 2 mmol/L glucose. All other test conditions contained addition of a fixed peptide concentration. Co-incubation with peptide ILAP (10^{-6} M) displayed 2 – fold GIP secretion, LLAP (10^{-6} M) 1.5 – fold secretion while peptide MAGVDHI (10^{-6} M) failed to promote any GIP secretion with 0.4 fold reduction in GIP secretion versus basal control, respectively (Fig 4.3 B).

Fig 4.3 C - Cellular toxicity of peptides during co-incubation in BRIN-BD11 cells

During acute insulinotropic experimentation supernatant from the highest concentration of peptides (10^{-6} M) were investigated for extracellular lactate dehydrogenase (LDH) concentration using the cytotox non-radioactive LDH assay. Each of the experimental

conditions investigated returned no-increase in LDH concentration over basal glucose control, respectively (Fig 4.3 C).

Fig 4.3 D - Acute intracellular cyclic adenosine monophosphate (cAMP) produced during acute peptide co-incubation in BRIN-BD11 cells.

Post-insulinotropic experimentation, BRIN-BD11 cells were further lysed and collected for cAMP concentration determination using a cAMP elisa assay (as described in 4.2.8). Results were comparative to basal glucose control (5.6 mmol/L) with positive controls, 16.7 mmol/L glucose and GLP-1 returning 20% and 30% increase in intracellular cAMP concentrations. Peptide ILAP and LLAP returned a 70% and 30% increase in intracellular cAMP concentration, however MAGVDHI displayed no increase versus basal control, respectively (Fig 4.3 D).

Fig 4.4 Investigating DPP-4 activity using a GLP-1 based RP-HPLC assay

DPP4 (5 mU) was co-incubated with GLP-1 (10^{-6} M) alone, or with the further addition of either Diprotin A (10^{-6} M), ILAP (10^{-6} M), LLAP (10^{-6} M), or MAGVDHI (10^{-6} M). GLP-1 alone in the presence of DPP4 resulted in 1.5 H half-life, whereas the known small peptide molecule DPP4 inhibitor Diprotin A resulted in a 24 hour half-life ($p < 0.001$). Subsequently the peptides ILAP, LLAP and MAGVDHI extended the GLP-1 half-life from 1.5 h to 8 h ($P < 0.001$, Fig 4.4 B), 9 h ($P < 0.001$, Fig 4.4 C) and 13 h ($P < 0.001$, Fig 4.4 D), respectively.

Fig 4.5 – 4.11 - Glucose lowering effect of peptides co-administered with glucose via intraperitoneal injection

Peptides were tested *in-vivo* via intraperitoneal injection. Blood glucose of each animal was measured prior to administration (0 min) with each peptide (25 nmol/kg bw) co-administered with glucose (18.8 mmol/kg bw) and subsequent blood glucose concentration measured at various timepoints (15, 30, 60, 90 & 120 min; Fig 4.5 - 9) with blood plasma collected for insulin RIA over a 2 hour period after administration. Blood glucose concentrations were reduced overall via analysis by area under the curve ($AUC_{\min120}$) after administration of both ILAP and LLAP with a blood glucose AUC reduction of 43-52% reduction ($P<0.05$; fig 4.8 A) and accompanied by a 2.9 to 4.4-fold rise ($p<0.01$; Fig 4.8 B) in plasma insulin ($AUC_{0-120\text{ min}}$, $P<0.01$ to $p<0.001$), compared to the glucose control. No improvement in glucose homeostasis was noted with MAGVDHI (Fig 4.8). When peptide LLAP and ILAP were co-administered with GLP-1 a further glucose reduction of 13% ($P<0.01$, Fig 4.9 B) and 16% ($P<0.01$, Fig 4.10 B) below GLP-1 injection alone when analysed via blood glucose area under the curve ($AUC_{0-120\text{ min}}$). No improvement was noted during the glucose challenge and co-administration of peptide MAGVDHI with and without GLP-1, respectively (Fig 4.11 B).

Fig 4.12 - Satiety promoting effects of marine derived peptides

Groups of mice ($n=8$) trained to eat for 3 H per day were administered peptides (25 nmol/kg bw) via intraperitoneal injection. Food was weighed with mice only allowed access to food post-injection. Food was weighed at 30 minute intervals up until 180 minutes. Peptide ILAP displayed a food intake reduction at 180 min only ($p<0.05$,) whereas peptide MAGVDHI reduced food intake from 90 min to 180 min ($p<0.05$ – $p<0.001$) versus saline control group. Peptide LLAP had no effect on food intake over the 180 minute period (Fig 4.12).

4.3 Results

Fig 4.1

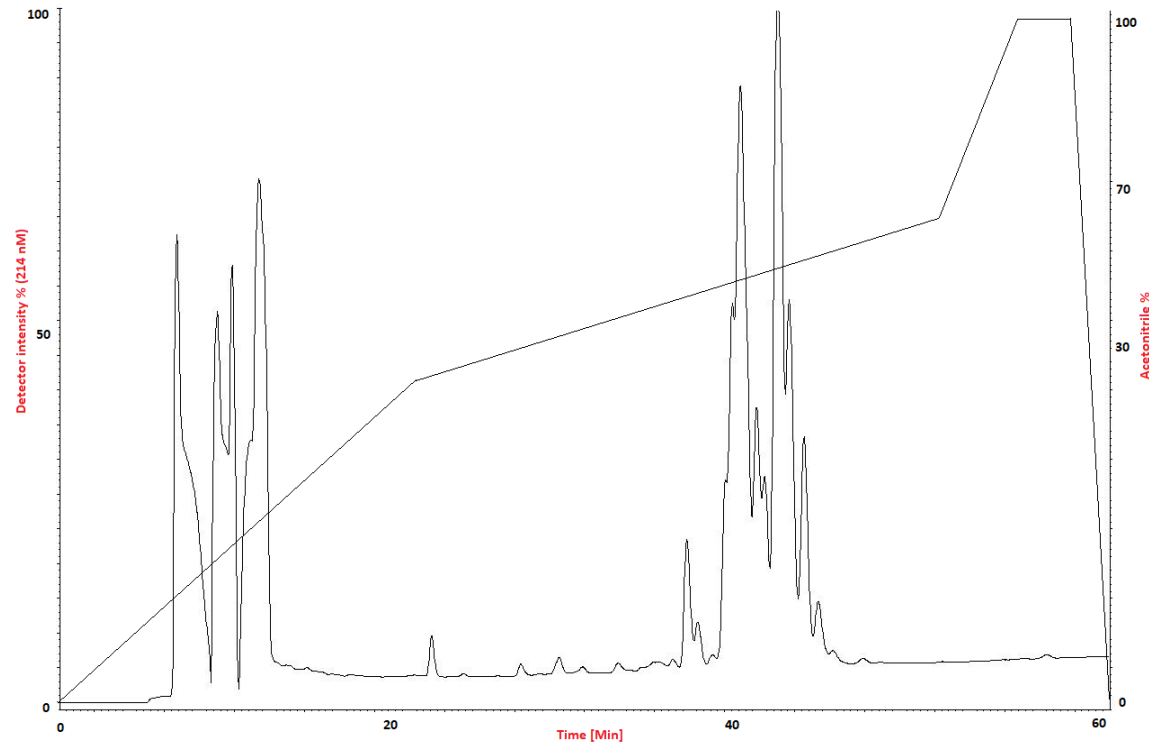


Fig 4.1 - A. Chromatograph displays a RP-HPLC elution of *palmaria palmata* hydrolysates generated using Alcalase and Flavourzyme combination (4h). Using a prep luna C18 column (250 x 10 mm, 10 μ m particle size) a 4 mg injection using a rheodyne 7725i injector attached to a thermo surveyor RP-HPLC machine equipped with a UV/VIS detector set to 214 nm wavelength. Solvent proportioning changed from dH₂O w/v 0.12% TFA to a maximum of 70% acetonitrile (70% acn, 30% dH₂O w/v 0.1% TFA) at a flow rate of 5 ml/min over 60 minutes.

Fig 4.2

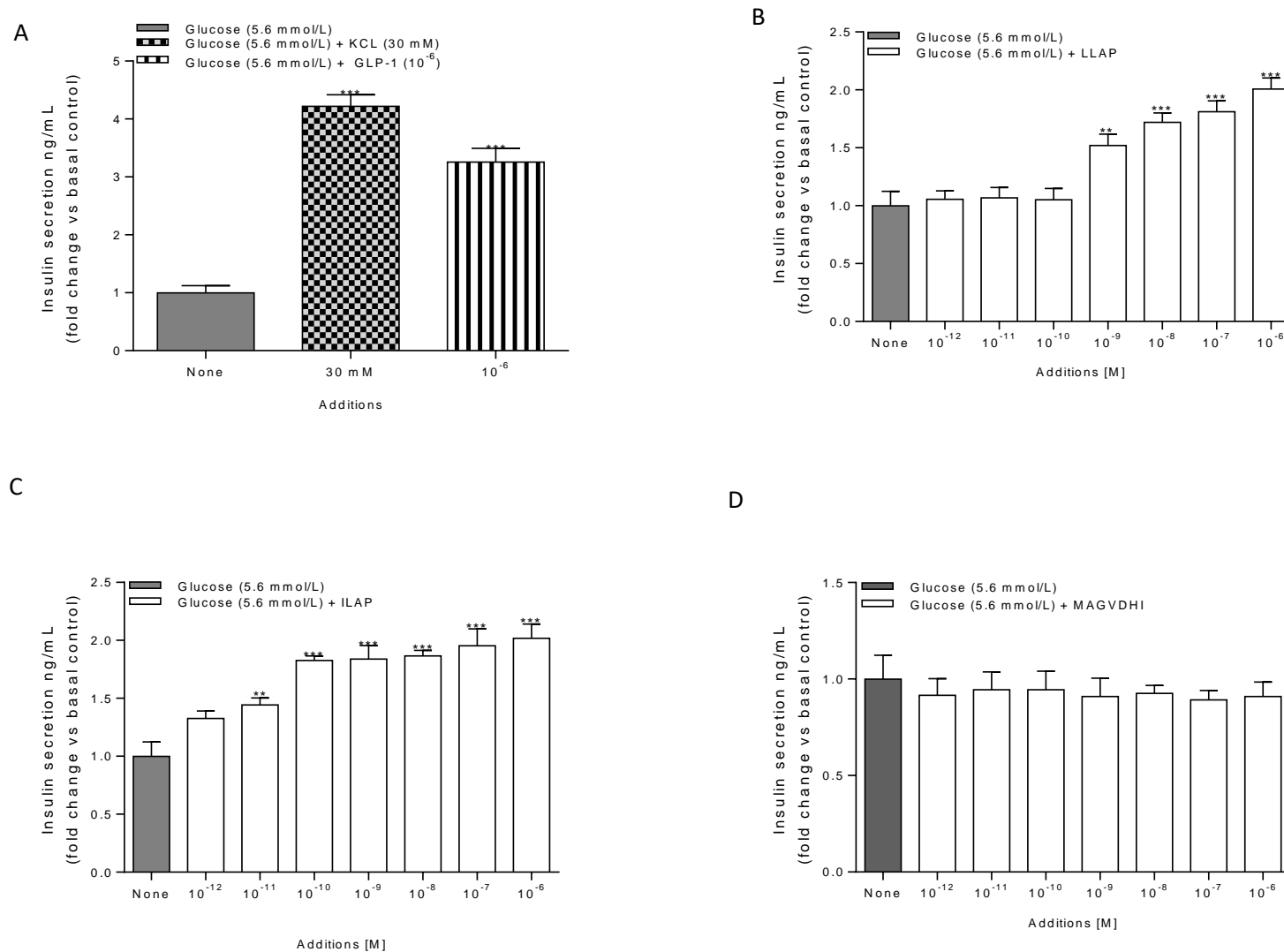
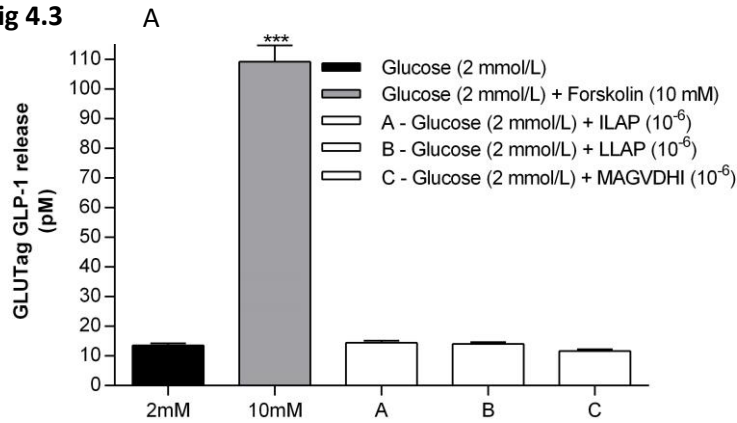
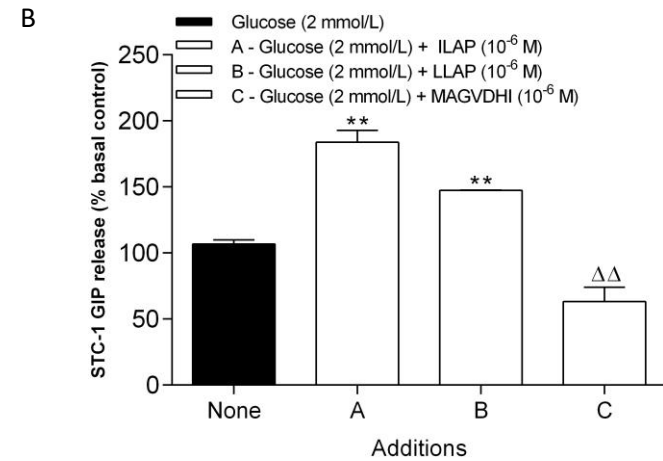


Fig 4.2. Dose-dependent effects of *Palmaria palmata* derived peptides on insulin secretion from BRIN-BD11 cells at 5.6 mM glucose. BRIN-BD11 cells were incubated with either LLAP (Panel B), ILAP (Panel C) or MAGVDHI (Panel D) using a range of concentrations (10^{-12} M to 10^{-6} M) for 20 min and insulin release measured by RIA. Values are expressed as mean \pm S.E.M. (n=8). * p <0.05, ** p <0.01, *** p <0.001 compared to respective glucose control. Panel A represents positive insulintropic controls used in the experiment.

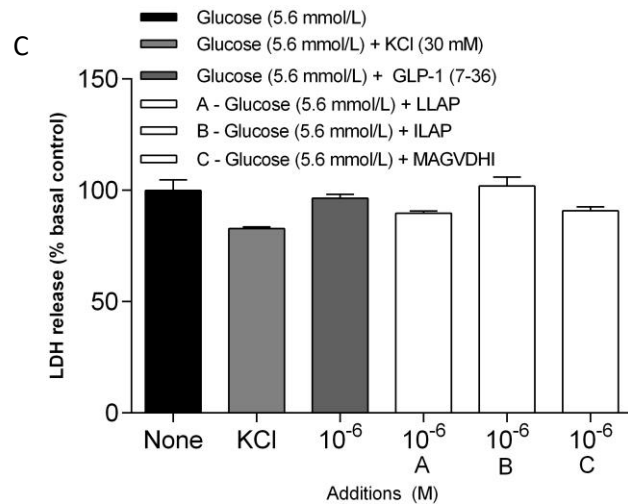
Fig 4.3



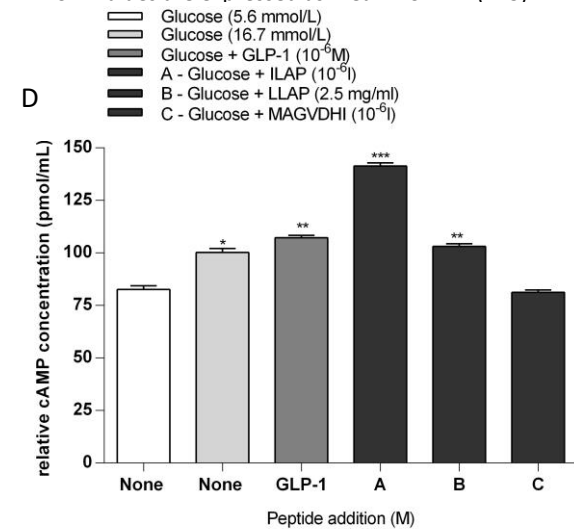
A. Secretory effects of peptides on GLP-1 secretion from GLUTag cells at 2 mM glucose. GLUTag cells were incubated with ILAP, LLAP or MAGVDHI using a fixed concentrations of peptide (10^{-6} M) for 2 hours and GLP-1 release measured by ELISA. Values are expressed as mean \pm S.E.M. (n=3).



B. Secretory effects of peptides on GIP secretion from STC-1 cells at 2 mM glucose. GLUTag cells were incubated with ILAP, LLAP or MAGVDHI using a fixed concentrations of peptide (10^{-6} M) for 2 hours and GIP release measured by ELISA. Values are expressed as mean \pm S.E.M. (n=3).



C. LDH release from BRIN-BD1 cells during co-incubation with ILAP, LLAP or MAGVDHI using a fixed concentrations of peptide (10^{-6} M) for 20 min with LDH measured by cytotox assay. Values are expressed as mean \pm S.E.M. (n=3).



D. Effects of marine peptides on cAMP production in BRIN-BD11 cells during acute co-incubation with glucose alone or ILAP, LLAP or MAGVDHI using a fixed concentrations of hydrolysate (10^{-6} M) for 20 min. Cells were lysed using cAMP assay lysis buffer with cAMP concentration established using a cAMP specific ELISA. Values are expressed as mean \pm S.E.M. (n=3). * p <0.05, ** p <0.01, *** p <0.001 compared to respective glucose only control.

Fig 4.4

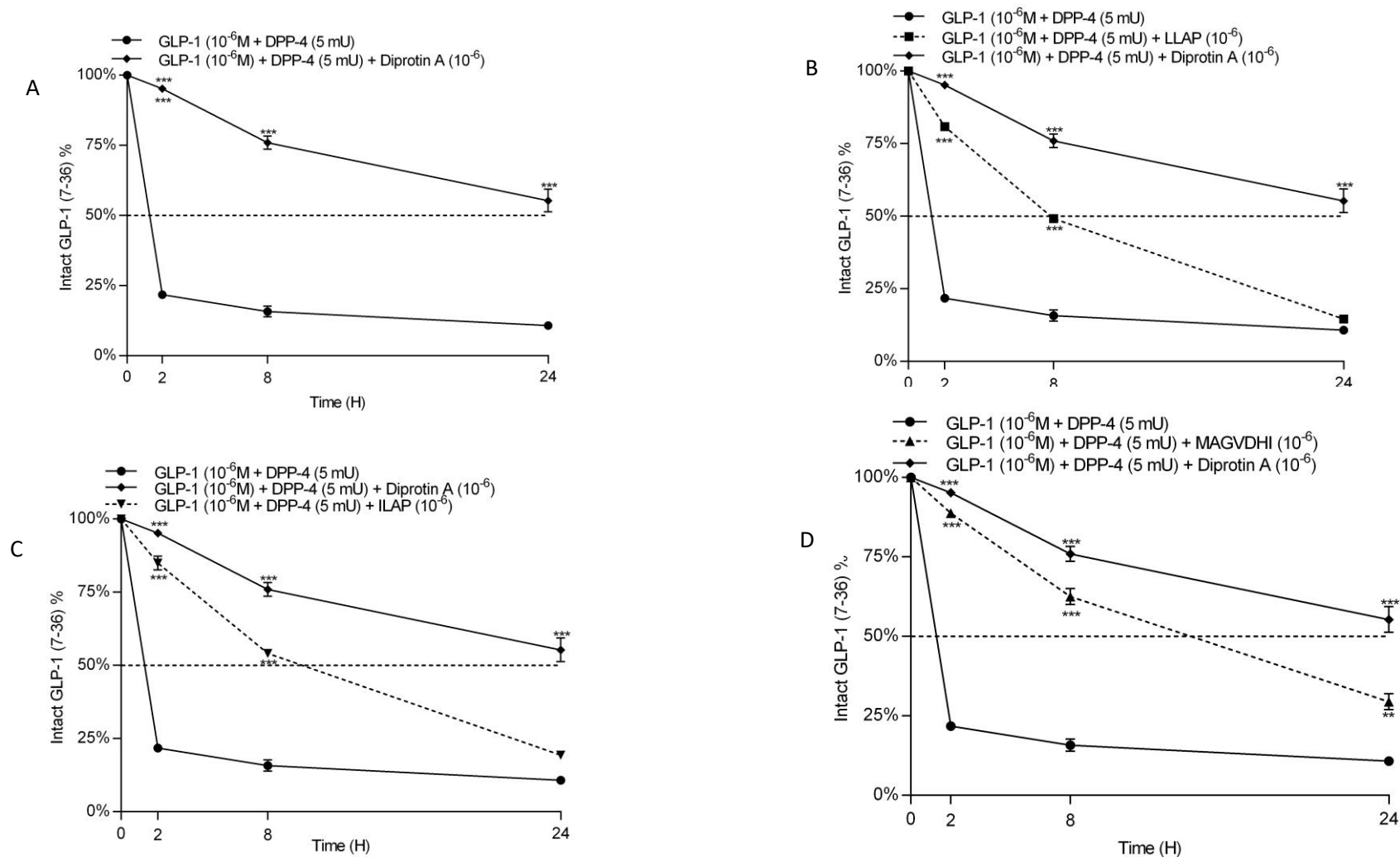


Fig 4.4 - Graphs displays a graphical depiction of a RP-HPLC elution of active GLP-1 (10⁻⁶ M^{7-36 amide}) after incubation with DPP4 (5 mU) at 0, 2, 8 and 24 H in a TEA buffer (pH 7.4). Using an analytical kinetix C18 column (250 x 10 mm, 3.6 μm particle size) a 200 μl injection using a rheodyne 7725i injector attached to a thermo surveyor RP-HPLC machine equipped with a UV/VIS detector set to 214 nm wavelength. **Panel A** represents GLP-1 and DPP4 only, **panel B** represented the addition of LLAP with **panel C** addition of ILAP and **panel D** displaying the addition of MAGVDHI. Each graph also contained the addition of Diprotin A as a positive control. Results are Mean±SEM for 2 separate HPLC elutions per timepoint. ***p<0.001 versus GLP-1 + DPP-4 alone.

Fig 4.5

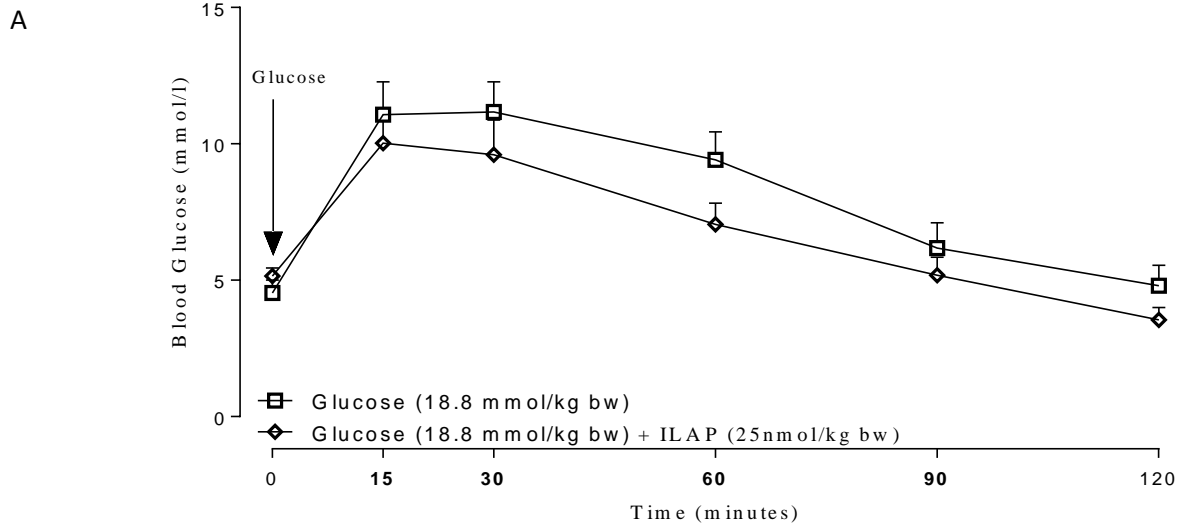


Fig 4.5 - A. Acute actions of ILAP on IP glucose tolerance in NIH Swiss mice. Glucose was measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; IP) or in combination with ILAP (25 nmol/kg body weight; IP). Values are expressed as mean \pm S.E.M. for 6 mice.

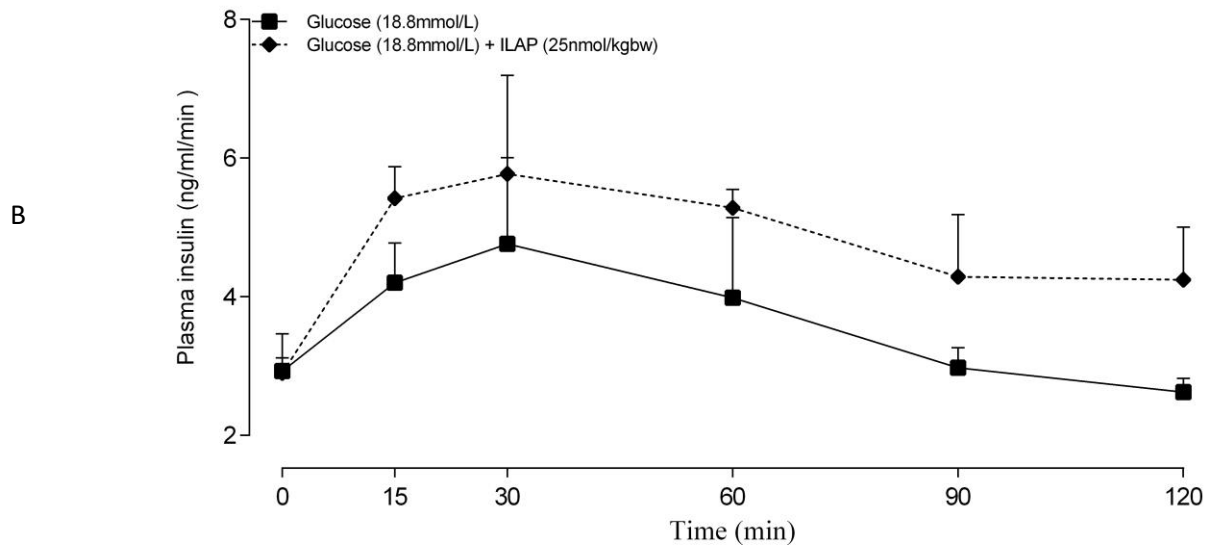


Fig 4.5 - B. Acute actions of ILAP on plasma insulin concentrations in NIH Swiss mice. Plasma insulin was measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; IP) or in combination with ILAP (25 nmol/kg body weight; IP). Values are expressed as mean \pm S.E.M. for 6 mice.

Fig 4.6

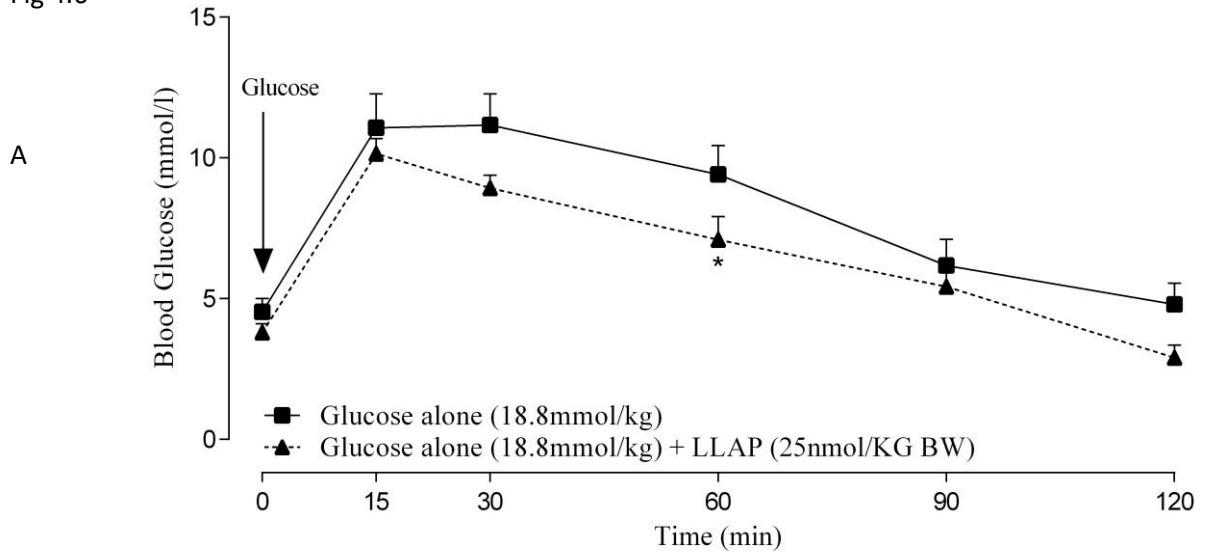


Fig 4.6 - A. Acute actions of LLAP on IP glucose tolerance in NIH Swiss mice. Glucose was measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; IP) or in combination with LLAP (25 nmol/kg body weight; IP). Values are expressed as mean \pm S.E.M. for 6 mice. * $p < 0.05$ versus glucose only control group

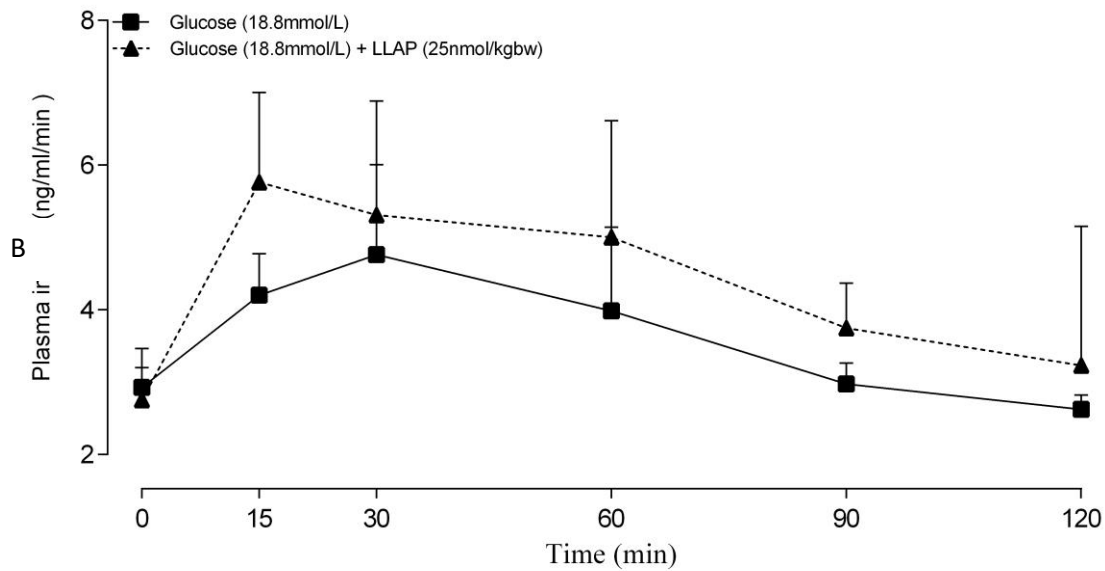


Fig 4.6 B. Acute actions of ILAP on plasma insulin concentrations in NIH Swiss mice. Plasma insulin was measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; IP) or in combination with LLAP (25 nmol/kg body weight; IP). Values are expressed as mean \pm S.E.M. for 6 mice.

Fig 4.7

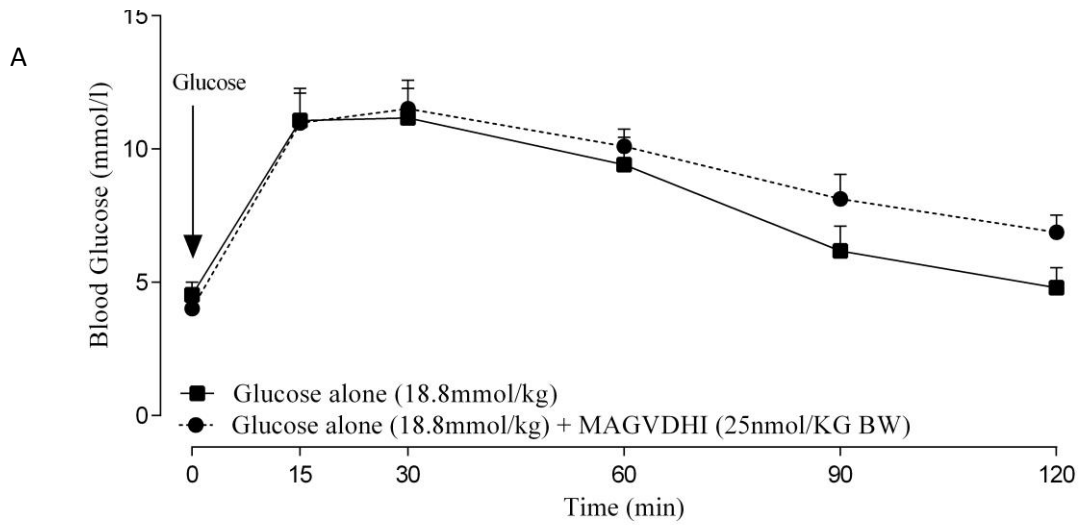


Fig 4.7 A. Acute actions of MAGVDHI on IP glucose tolerance in NIH Swiss mice. Glucose was measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; IP) or in combination with MAGVDHI (25 nmol/kg body weight; IP). Values are expressed as mean \pm S.E.M. for 6 mice.

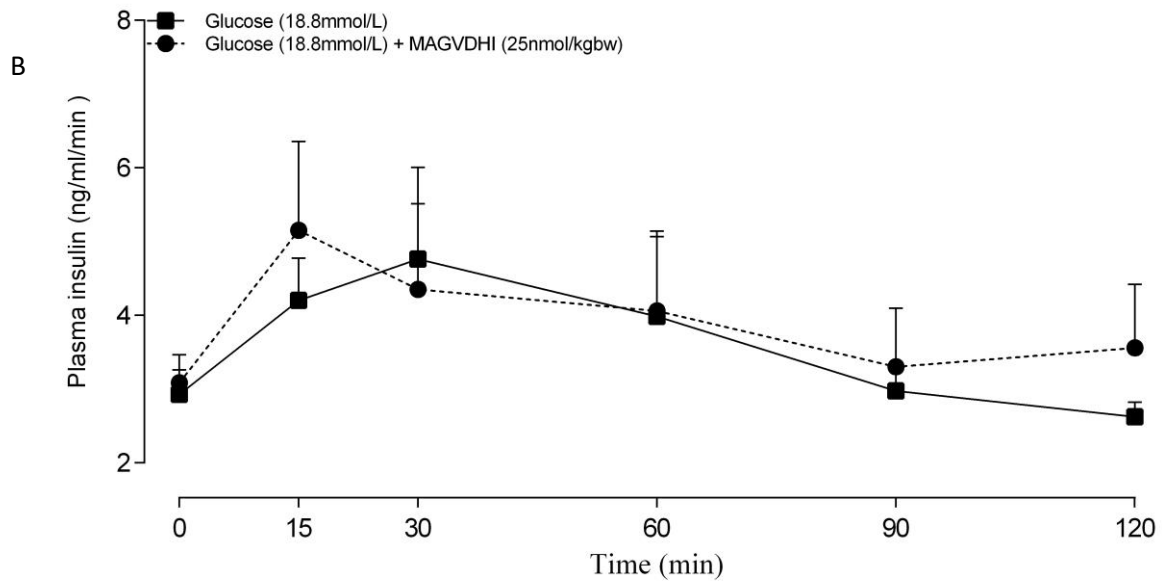


Fig 4.7 B. Acute actions of MAGVDHI on plasma insulin concentrations in NIH Swiss mice. Plasma insulin was measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; IP) or in combination with MAGVDHI (25 nmol/kg body weight; IP). Values are expressed as mean \pm S.E.M. for 6 mice.

Fig 4.8

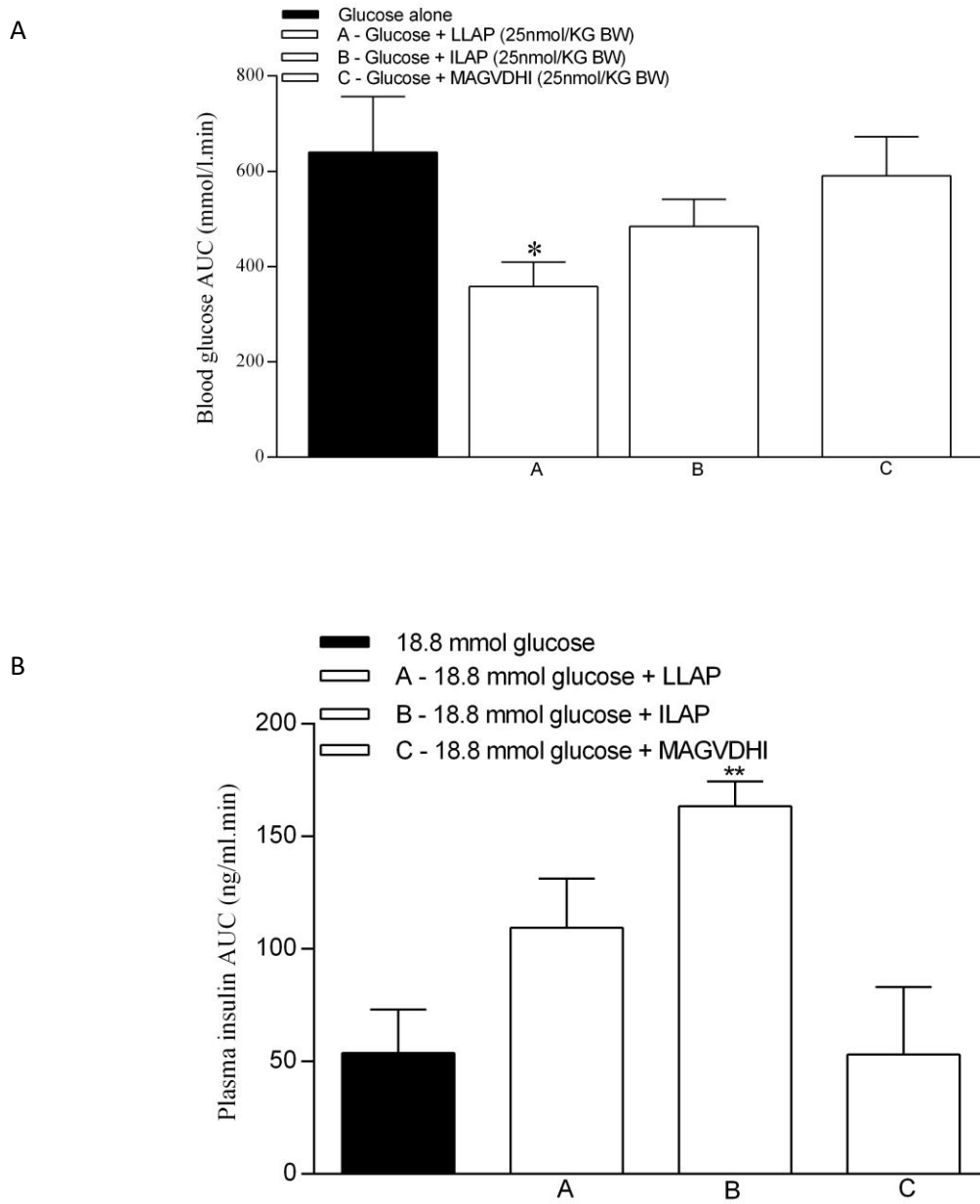


Fig 4.8 A/B. AUC values for 0-120 min post-injection compared to glucose alone with panel G representing blood glucose and panel H representing insulin total AUC values. Values are expressed as total \pm S.E.M. for 6 mice. * $p < 0.05$

Fig 4.9

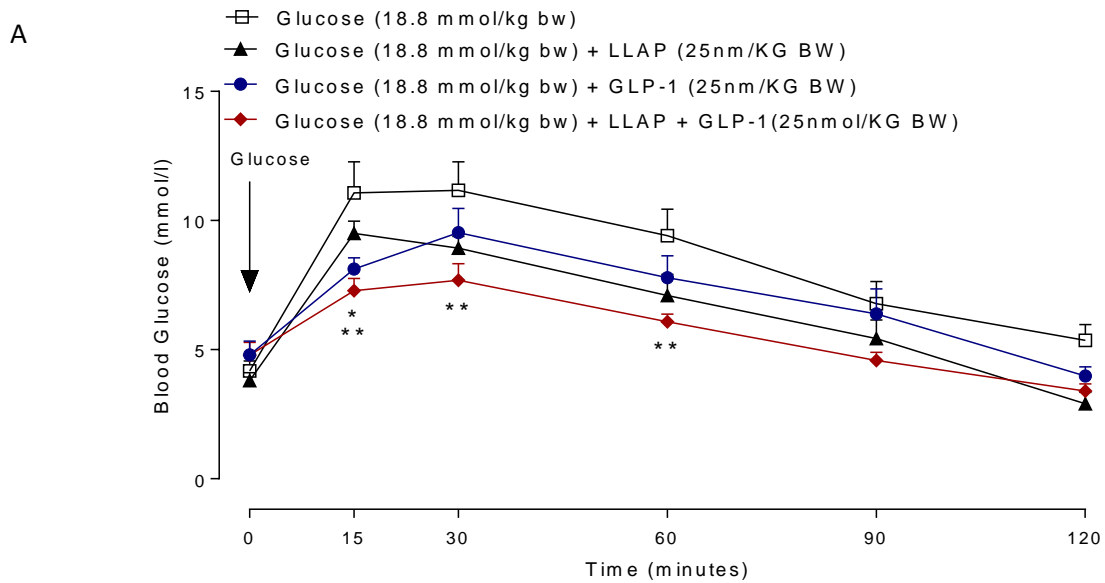


Fig 4.9 A. Acute actions of LLAP on IP glucose tolerance in NIH Swiss mice. Glucose was measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; IP) or in combination with LLAP with and without GLP-1 in combination(25 nmol/kg body weight; IP). Values are expressed as mean \pm S.E.M. for 6 mice.

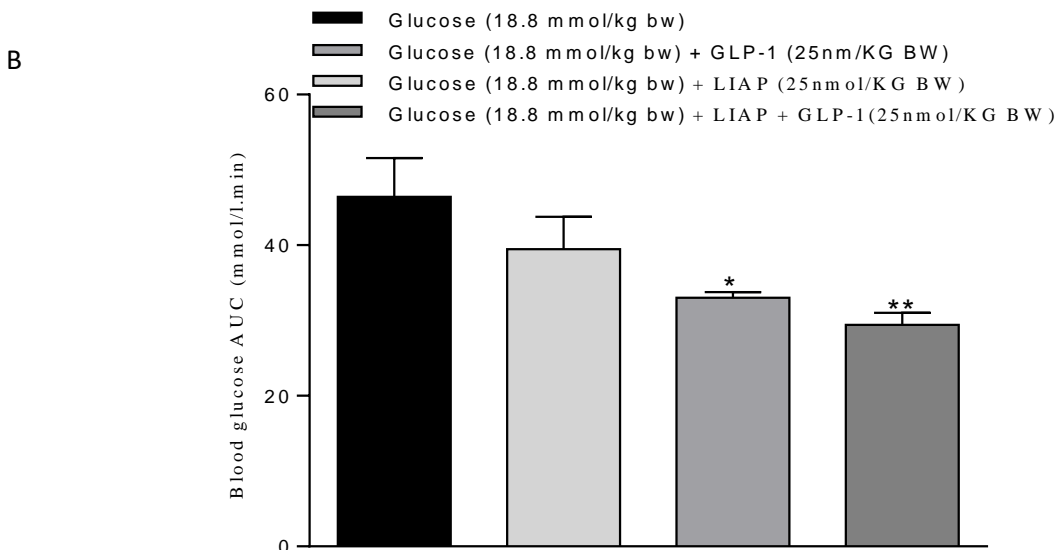


Fig 4.9 B. Mean AUC values for 0-120 min post-injection compared to glucose alone with panel B representing blood glucose AUC values. Values are expressed as total \pm S.E.M. for 6 mice. *p<0.05

Fig 4.10

A

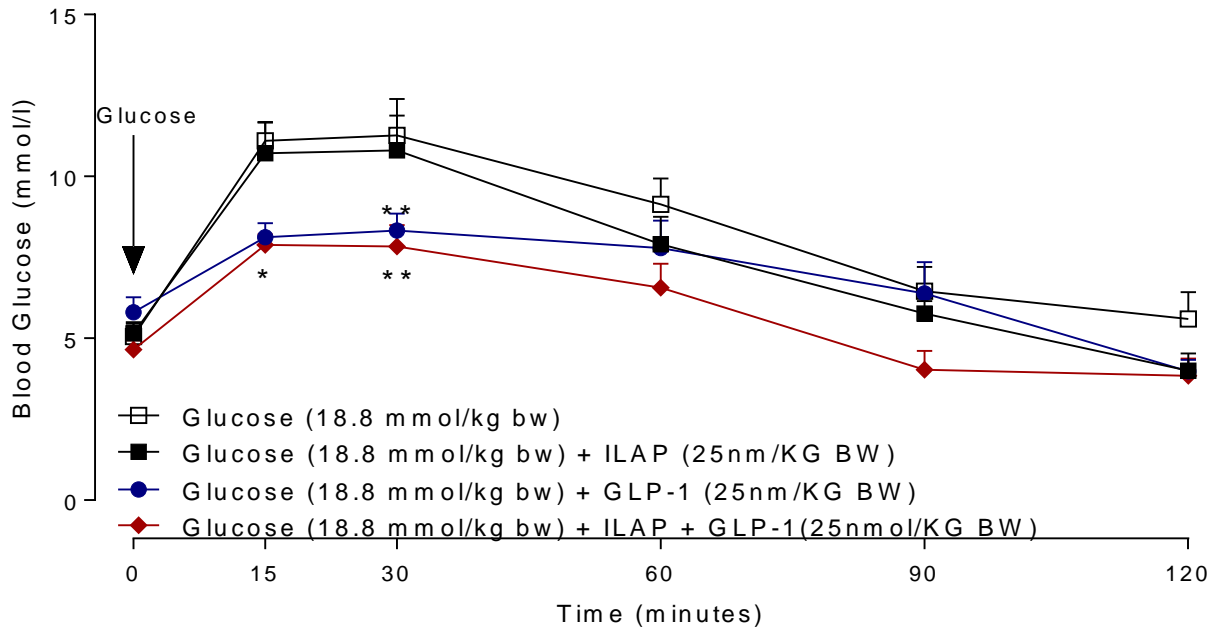


Fig 4.10 A. Acute actions of ILAP on IP glucose tolerance in NIH Swiss mice. Glucose was measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; IP) or in combination with ILAP with and without GLP-1 in combination (25 nmol/kg body weight; IP). Values are expressed as mean \pm S.E.M. for 6 mice.

B

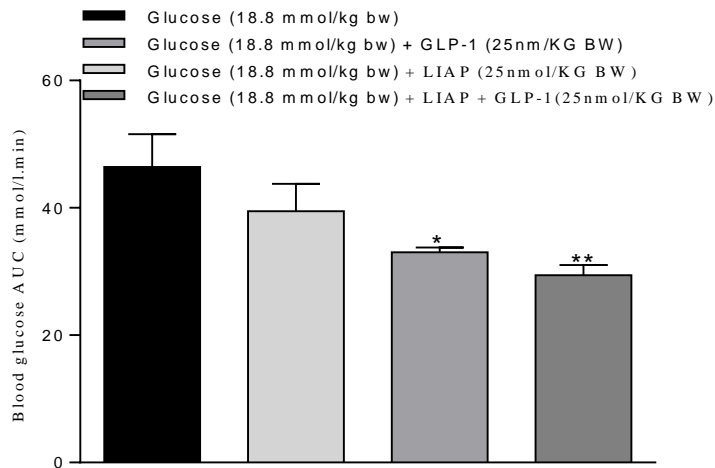


Fig 4.10 B. Mean AUC values for 0-120 min post-injection compared to glucose alone with panel D representing blood glucose AUC values. Values are expressed as total \pm S.E.M. for 6 mice. *p<0.05

Fig 4.11

A

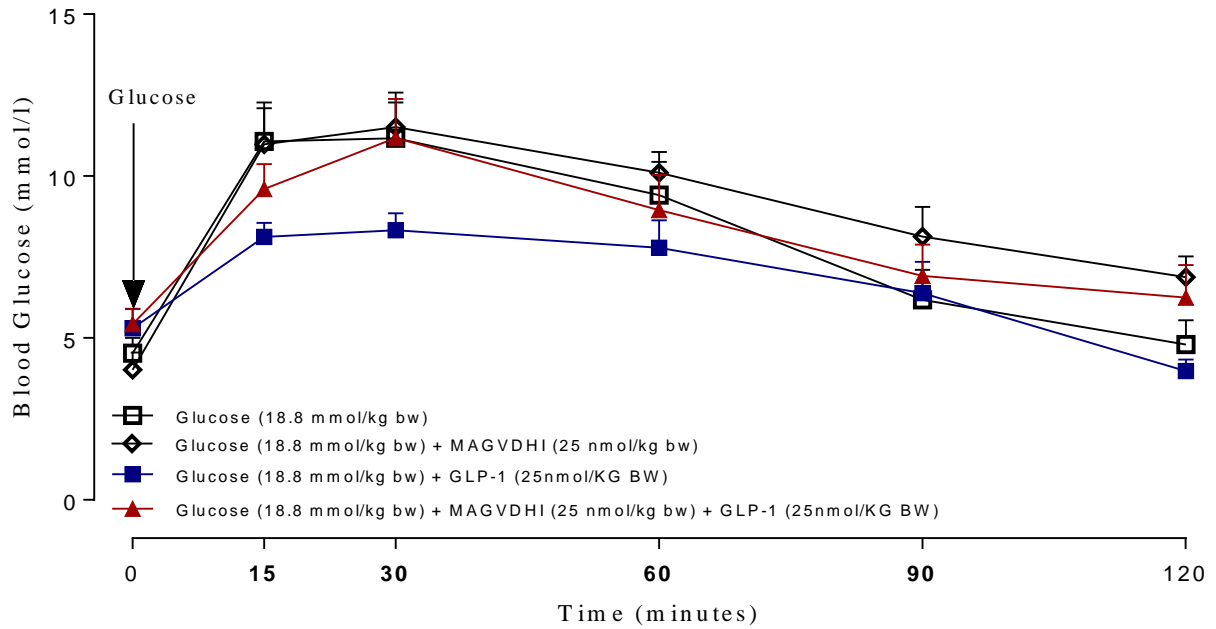


Fig 4.11 A. Acute actions of MAGVDHI on IP glucose tolerance in NIH Swiss mice. Glucose was measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; IP) or in combination with ILAP with and without GLP-1 in combination(25 nmol/kg body weight; IP). Values are expressed as mean \pm S.E.M. for 6 mice.

B

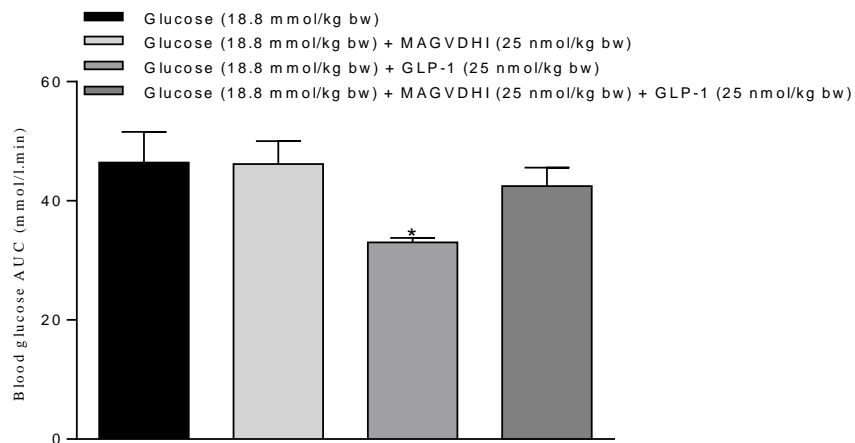


Fig 4.11 B. Mean AUC values for 0-120 min post-injection compared to glucose alone with panel D representing blood glucose AUC values. Values are expressed as total \pm S.E.M. for 6 mice. *p<0.05

Fig 4.12

A

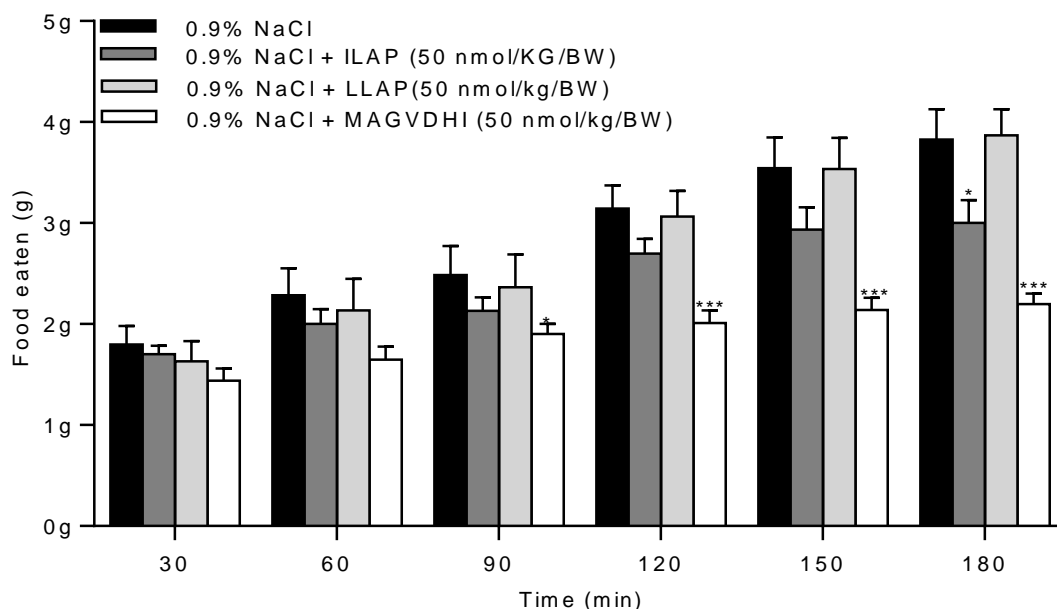


Fig 4.12. Food intake data for mice with restricted food availability (3 hours per day). Each group was given an IP injection with a compound of interest. A weighed amount of food is given (T 0 min) and amount eaten weighted every 30 minutes for 180 minutes. Marine peptides were administered via IP, (ILAP, LLAP and MAGVDHI) with food eaten calculated accumulatively and comparative to non-treated saline control. Panel C represents oral palmaria palmata hydrolysates and panel D, boarfish hydrolysates effect on food intake. Values are expressed as mean \pm S.E.M. for 8 mice. * $p < 0.05$ ** $p < 0.01$ compared to glucose alone.

Summary of analysis

In vitro				
Peptide	Fold increase of insulin secretion versus basal control	Lowest concentration needed to promote insulin secretion in BRIN-BD11 Cells	Fold increase of GLP-1 secretion versus basal control from GLUTag cells	GLP1 + DPP4 longevity duration versus control
LLAP	2-fold ($p < 0.005$)	10-9 M ($p < 0.01$)	*ns	8 h ($p < 0.001$)
ILAP	2-fold ($p < 0.005$)	10-11 M ($p < 0.01$)	*ns	10 h ($p < 0.001$)
MAGVDHI	*ns	*ns	*ns	14 h ($p < 0.001$)
In vivo				
Peptide	Reduction in post-prandial glucose AUC following IP injection	Increase in post-prandial insulin secretion AUC following IP injection	Additive post-prandial glucose reduction when combined with GLP-1 following IP injection	A reduction in food intake displayed following IP injection
LLAP	Yes ($p < 0.05$)	*ns	Yes ($p < 0.01$)	180 min ($p < 0.05$)
ILAP	*ns	Yes ($p < 0.01$)	Yes ($p < 0.01$)	*ns
MAGVDHI	*ns	*ns	*ns	90 min ($p < 0.001$)

4.4 Discussion

To date there has been a wealth of knowledge applied to the field of potential therapies based upon peptides, which display antidiabetic properties (Billyard *et al*, 2007). This applies to classical and more recently identified approaches that aim to exploit homeostatic mechanisms, which in turn has concomitant application for improved health and physiological regulation. Diabetes research is firmly established in developing new approaches of which regulate endogenous hormones or more recently the exploitation of existing therapeutic pathways for medicinal advantage (Todd & Bloom, 2007). Due to the rapid global rise of type 2 diabetes, approaches have shifted from classical lifestyle alterations and the well-established biguanide and sulphonylurea treatments targeting pancreatic and hepatic cell signalling to peptide based approaches which have shown sustained improvement of pancreatic functioning (Lau & Dunn, 2018). The utilization of peptides of all classes have evolved and changed dramatically, including peptides isolated from natural sources. New therapies such as DPP4 inhibition mentioned earlier of which lengthens the post-prandial incretin effect. This in turn allows hormones such as GLP-1 and GIP to cause potent glucose dependent insulin secretion, thus lowering overall circulating plasma glucose concentrations. Furthermore, hormones of interest have over the past decade diversified and now include interest in numerous gut related hormones such as leptin, ghrelin, peptide YY, obestatin and oxyntomodulin to name a few (Sala *et al*, 2014).

Overall, these approaches of exploiting the later hormones (excluding DPP4 and GLP-1 therapies) of which may promote improved glucose, insulin and glucagon homeostasis has yet to fully materialise and opens the questions to researchers, what prompts the intestines, or specifically, certain intestinal cells to fail to counteract the rapid increase in global hyperglycaemia?. This chapter attempts to expose the multifaceted properties of identified peptides derived from a protein source of which was once a staple of the Irish and European

diet, *Palmaria palmata*. Each of the assays deployed aim to expand on the crude hydrolysates results presented in the previous chapter. As such verging from the crude hydrolysate co-incubation to the identified peptides, ILAP, LLAP and MAGVDHI presented interesting results within the *in vitro* assays and *in vivo* animal investigation. Bioactive peptides from seaweed protein in the past was unfortunately not given the attention it require, and more recently taken forefront towards increasing sustainability of the nitrogen source (Admassu *et al*, 2018).

Firstly, elaborating on previous crude work carried out the same screening approach was taken using the insulin secreting BRIN-BD11 cell line with each of the peptides investigated for bioactivity and potency. Initially, MAGVDHI displayed no increase in basal insulin secretion, interestingly peptide ILAP stimulatory effect was below 10^{-9} M was not above basal glucose control whereas a single amino acid change in the form of ILAP resulted in similar secretory potency from 10^{-6} M through to 10^{-10} M and as low as 10^{-11} M still resulting in elevated insulin secretion. The interest in small molecule receptor agonists is at the forefront of diabetes research with particular interest with incretin specific receptor activation such as the GLP-1 receptor (Willard *et al*, 2012). Its unclear if the insulinotropic activity of LLAP and ILAP are receptor based, however the increased cAMP reported would suggest a downstream cascade effect which is likely due to membrane based activation, however further work is needed to elucidate the mechanism of action.

Furthermore, the insulin secretory effect of these synthetic peptides is lower compared to its crude hydrolysate counterpart of which they were identified suggesting a synergistic effect of multiple peptide compounds within the crude hydrolysate, however ILAP appears to be insulinotropic at considerably lower concentrations that LLAP and MAGVDHI. Rationale for this may arise from the protease used bromelain. Bromelains cleavage site converts protein carboxyl groups into uncharged amides which can impede effect towards cellular based receptors. Furthermore, secretory investigation using GLP-1 producing GLUTag

cells, each of the peptides failed to stimulate GLP-1 secretion above the basal glucose control, again adding to the hypothesis that cellular receptor/peptide interaction may be hindered using this digestion process. With this being noted, ILAP and LLAP, similar to the secretory activity within the BRIN-BD11 cell line, resulted in elevated GIP secretion within STC-1 cells, whereas fascinatingly, MAGVDHI inhibited the relative GIP secretion versus the basal glucose control; however, the reason for this is unknown. Oral protein intake is considered beneficial due to the incretin effect which may have beneficial glucose lowering effects in type 2 diabetes (Jakubowicz *et al*, 2014). With this noted, as these peptides were injected rather than orally administered, their insulinotropic effect may be derived from the pancreas.

The toxicity profile of these peptides returned favourable results within eukaryotic cells. The immunogenicity of peptide-based therapeutics is a cause for concern; however, no adverse effects were noted during acute co-incubation with the algal derived peptides (Gupta *et al*, 2015). During acute intracellular signalling investigation, cAMP concentration was established after acute incubation within culture cells; interestingly ILAP presented a potent rise in intracellular cAMP production with LLAP resulting in similar levels to that of GLP-1. The activation of cAMP dependent pathways may shed light on why both ILAP and LLAP presented secretory activity in both BRIN-BD11 and STC-1 cells, as under the same experimental conditions MAGVDHI resulted in the same intracellular cAMP as the basal glucose control. This result would correspond with, up until now, the cellular secretory pattern seen with peptide MAGVDHI, as GLUTag and STC-1 trigger hormone secretion from cellular cAMP signalling pathways being activated prior to hormone exocytosis concluding that MAGVDHI is relative inert towards cellular based receptors (Simpson *et al*, 2007).

Protein-protein interaction was investigated using an RP-HPLC assay approach. Under the experimental conditions, GLP-1 and DPP-4 incubated alone yielded a 1.5 h half-life of the active form of GLP-1 over the 24 hour period. Subsequently, after the addition of each peptide

the half-life of GLP-1 was significantly extended across all three peptides of interest, this time highlighting that MAGVDHI using this approach was successful at inhibiting the action of DPP4 resulting in a 13 h half-life versus 8 h and 10 h for peptides LLAP and ILAP, respectively. These algal peptides have been previously reported within the literature for their DPP-4 inhibitory action (Harnedy *et al*, 2015). However, with this noted, the study mentioned that peptides with ile at the penultimate position having a negative effect on DPP4 activity. Our own work corroborated this finding, furthermore we confirmed that the mechanism of which MAGVDHI inhibits DPP4 is unknown at present, however we hypothesize that the intact peptide chain may not itself present the inhibitory activity seen, more-so its further degraded amide bonds that may be released during DPP4 interaction.

During IPGTT investigation, we identified in the previous chapters that therapeutic gain from crude peptides might be exhibited primarily via the gut, however with peptides ILAP and LLAP eluding effects upon cultured pancreatic cells, that the delivery of the identified synthetic marine peptides may also present glucose lowering effects via intraperitoneal (IP) injection. This method aimed to assess that bypassing the intrinsic intestinal signalling via post-prandial pathways may also be advantageous for glucose regulation in the fed state such as that of current peptide mimetics via injection of GLP-1 mimetics (Gupta, 2013). Delivery of peptides via injection avoids the harsh proteolytic intestinal environment. In order to test this approach, mice were given an IP glucose challenge including 25 nmol/kg/bw of each peptide to assess their efficacy as potential anti-diabetic peptides. Interestingly, after injection, overall plasma glucose was lower post-injection when analysed via area under the curve over the 2 H period in both ILAP and LLAP groups with an increase in circulating plasma insulin during the same investigation period. It was unclear if the mechanism of which circulating insulin was elevated, either via DPP-4 inhibition and improvement in GLP-1 action upon the pancreas, or direct pancreatic interaction and promotion of insulin secretion. Expanding on the knowledge that these peptides capable of inhibiting DPP4, displayed here and previous studies, co-

injection of peptide ILAP or LLAP with GLP-1 further improved the glucose lowering effect versus the GLP-1 only injected group, when challenged via the same glucose concentration; however, improvement beyond GLP-1 only was marginal when analysed via AUC. The *in-vitro* investigating displayed a lack of activity for peptide MAGVDHI, exceptions being the decrease in DPP4 activity via RP-HPLC assessment; furthermore, no improvement in glucose homeostasis was seen *in-vivo* during the IPGTT of MAGVDHI alone or in combination with GLP-1. Blood glucose levels after the IPGTT however increased in the latter experiment when co-injected with GLP-1, eluding to the peptides potential antagonistic effect upon biological pathways. This antagonistic effect was presented within the STC-1 cells and lack of secretory effect upon BRIN-BD11 and GLUTag cells, respectively.

Finally, groups of trained mice of which had access to food for 3 H per day were used to assess the effect upon food intake during food availability at 30 min intervals. Concluding this, peptide LLAP had no effect upon food intake, whereas ILAP presented a reduction at the 180 min time point only. Interestingly, peptide MAGVDI resulted in a significant reduction in food intake from 90 min through 180 min with overall food reduction across the entire group of mice averaging 45% versus the control group. In order to assess the rationale for this we must assume the peptides ILAP and LLAP are true DPP4 inhibitors due to their lack of effect upon food intake. Dietary protein intake and improvement in satiety via a reduction in food intake is commonly reported (Paddon-Jones, 2008), however injection of peptide based molecules and their effect on food intake is less known towards satiety.

Conclusion

Identifying several novel peptide hormones of which present multifaceted effects upon cultured cells, ability to elevate intrinsic intracellular signalling mechanisms, interact with large complex endogenous enzymes and finally improve glucose homeostasis and promote satiety in mice has added greatly to the overall hypothesis of this work that protein in its

hydrolysed form can elicit effects that have more than a purely nutritional role. Protein is vital for life; however the form of which it is ingested may alter the therapeutic effects displayed. Here we presented three peptides hormones of which were identified from a marine protein source, *Palmaria palmata*. On completion of the protein isolation, food grade enzymes were deployed to yield abundant small molecular weight peptides of which were subsequently separated, identified and synthesized for investigational purposes. Each peptide presented unique interactions upon *in-vitro* and *in-vivo* experimentation, which concluded that while the protein source was identical, each individual peptide may present unique therapeutic effects of which might be exploited for medical gain. Concluding this work, the research agrees that ILAP and LLAP are DPP4 inhibitors, which also elicit additional properties, most likely dependent on its cellular surroundings. Peptide MAGVDHI however will need further characterisation to fully elucidate its potential as a satiety promoting peptide. Each of the peptides individually however, are modest DPP4 inhibitors comparative to synthetic molecules presently prescribed to treat type 2 diabetes, however this does not neglect the findings that these are food derived molecules with high safety and tolerance profiles and incorporation of the crude protein hydrolysate of which they were identified may prove beneficial for the treatment of type 2 diabetes and obesity.

Chapter 5

Glucose lowering, insulinotropic and satiety promoting effects during oral intake of marine protein hydrolysates using *in-vivo* animal models

5.1 - Introduction

The overwhelming rise of obesity related diabetes and associated co-morbidities has in recent years presented one of the greatest public health threats to both developed and the developing world (Wang, 2012; Ng *et al*, 2014). Increased rates of morbidity and mortality have unearthed further personal, economic and societal outcomes with devastating impact on health related wellbeing, thus there is an urgent need for direct and effective tools to prevent weight gain within the population and approaches to maintain weight loss (Lenz *et al*, 2009).

Non-insulin dependent diabetes mellitus is the most common obesity related endocrine disorder expected to affect more than 640 million people by 2040 (Marín-Peñalver *et al*, 2016). To date, classical approaches of controlling type 2 diabetes via conventional weight loss using lifestyle modifications with the aim of successful weight and hormonal homeostasis is rapidly surpassed in favour of intensive pharmacological strategies due to insulin resistance, weight loss plateau and the progression of diabetes complications (Bramante *et al*, 2017). Long term weight loss for treating obesity related diabetes unfortunately have a low successes rate at higher body mass indexes (BMI) (Monteso *et al*, 2016), however with nutritional guidance dramatically improves diabetes disease-associated outcomes. Western food environments are currently dominated by energy dense, highly processed and relatively inexpensive foods (Mattes & Foster, 2014), however a modest 10% weight loss can significantly improve obesity related health outcomes (Van Gaal & Scheen, 2015).

Furthermore, the only sustainable weight loss approach in the super-morbidly obese is achieved by gastrointestinal surgery in the form of gastric sleeve, bypass, banding or balloon (Golzarand *et al*, 2017). Researchers have now questioned the importance of the of the intestine as surgery procedures, such as the Laparoscopic Roux-en-Y gastric bypass (RYGBP), completely reverses type 2 diabetes in almost 80% of cases well before significant weight loss is displayed. This improvement is initially due to improvements in insulin sensitivity and metabolic improvements that are considered humoral in nature (Pajacki *et al*, 2007; Goldfine & Patti, 2014).

Gut hormone changes are noted, specifically with foregut surgery, which decreases the appetite promoting hormone ghrelin while increasing post-operative cholecystokinin (CCK) levels while hindgut surgery advances GLP-1 and PYY secretion (Beckman *et al*, 2010). The importance of protein, carbohydrates and lipid ratios within foodstuffs for weight management has been relatively consistent within the literature; defining that reduction total caloric intake can ultimately, lead to improvement in weight associated disease outcomes (Finer, 2001). Conversely, high protein intake in childhood and adolescents can be a factor leading to childhood obesity; however, protein intake is generally higher across many ages ranges leaving the direct association of the protein source and weight gain challenging to elucidate (Del Mar Bibiloni *et al*, 2015; Wallace & Frankenfeld, 2017). This association is a puzzling primarily due to the associated complexities of macronutrients within the foodstuff that ultimately need to be factored in. Controversial outcomes has unfortunately left researchers shy to fully clarify the importance of protein including the source which may display more than a caloric role for health and disease via the use of large scale studies with dietary adherence.

Nutritional promotion of humoral factors and have proved invaluable for understanding the mechanisms for ameliorating glycaemic and appetite control. Satiating factors via low glycaemic index foods or hormonal promotion of CCK, PYY, GLP-1, GIP and leptin have reported concurrent glucose lowering capabilities via satiation, reduction of gastric emptying or direct stimulation of insulin secretion via β -cell activation (Anderson & Woodend, 2003; Klok *et al*, 2007; Cheung *et al*, 2009; Gault *et al*, 2011; Joshi *et al*, 2013).

In overweight and obese patients with established T2DM, glucose homeostasis is achievable prior to weight loss with recent advances in hormone alteration via endogenous highly specific molecules, such as DPP-4 and SGLT2 inhibitors or GLP-1 mimetics (Chaudhury *et al*, 2017). Obesity and type 2 diabetes presents abnormal hormones concentrations such as cholecystokinin (CCK), leptin, ghrelin, adiponectin and peptide tyrosine-tyrosine (PYY) (Breen *et al*, 2013). Several rapid biochemical changes occur following intestinal bypass surgery; however, these hormonal changes are acutely amendable via post-prandial food intake (Coll *et al*, 2007). As such over several decades of nutritional research has highlighted this as a potential therapeutically viable approach to identifying foods and food components, which may act as vital glycaemic, appetite and metabolic regulators.

Oral intake of several sources of protein involve interaction with gut hormone secreting cells are directly involved in improved β -cell functionality, satiety, reduction of gastric emptying and insulin secretion, It is not clear however, the source or quantity needed of protein that leads to this outcome (Jakubowicz *et al*, 2014). Peak plasma amino acid circulation is considered the classical approach for describing protein metabolism, furthermore several decades of intestinal signalling and transport research has identified many cells, which possess specific membrane transport systems for peptides (Daniel, 2004).

Protein administration however has several major limitations; firstly, of the administered protein of interest, which aspect of the protein or peptides display the beneficial effect and secondly, during digestion of the protein, which degraded hydrolysate product remains bioactive after oral ingestion? Albeit, extraction of protein from diverse biological sources has reaffirmed that protein possess beneficial biological properties that are yet to be fully understood.

Proteins sources including cod ,salmon, herring and blue whiting are several species of ichthyoids of which present promising nutraceutical, anti-obesity and anti-diabetic potential (Hoyle & Merritt, 1994; Vik *et al*, 2015 Godinho *et al*, 2016; Harnedy *et al*, 2018). Protein extracted and hydrolysed from these sources have offered the ability to ameliorate weight gain and increase insulin sensitivity while promoting satiety, anti-inflammatory and lower adiposity *in vivo*. Our own hydrolysate research displayed potent insulin, GLP-1 and GIP secretion and finally improved glucose uptake in cultured adipocytes from a range of hydrolysates from marine sources, however bioactivity between each protein source varied leaving an understanding that the source of protein is a leading factor for potential targeted therapeutic effect. This work aims to answer if the source of protein and hydrolysis method play a role in the biological effect using altered doses of marine derived protein hydrolysates. Each of which, will be investigated for their effect upon glucose tolerance during acute and delayed glucose tolerance tests including effect upon food intake in mice.

Background & Aims

Within the literature, the optimal dose required to promote a positive effect from oral intake of protein ranges widely. Furthermore, the timing of the protein intake accompanied with

effect upon glucose lowering or satiety also vary. Unfortunately, a large proportion of the currently literature focuses on dietary protein sources from food stuffs or in the form of supplemental intact proteins such as those derived from dairy or plant based sources. For this reason, this chapter aims to identify the optimal dose required to promote a beneficial effect in regards to post-prandial glucose excursion, such as blood glucose levels pre- and post-oral administration of protein hydrolysates, including the delayed anti-glycaemic effect. Finally, the effect upon satiety in mice trained to eat for 3 hours per day using a similar protein concentration that promoted a reduction in post-prandial glucose elevation will be deployed. With this noted the translation of bioactivity from *in vitro* to *in vivo* study would be a main investigative outcome. The hypothesis for this chapter is in line with current literature of which is in agreement with protein promoting both direct and delayed improvements in post-prandial glucose excursion and a positive effect upon satiety and food intake, however the dose required and from which biological source will promote the most favourable effect is unknown presently.

5.2 Materials and methods

Acute glucose tolerance screening of marine protein hydrolysates

ice were grouped depending on their weight and non-fasting blood glucose concentration. Drinking water and standard rodent diet was freely available. All animal studies were performed in accordance with the UK Animals (Scientific Procedures) Act 1986. For experimental and animal information refer to section 2.4.3.

Glucose tolerance tests

Blood samples were measured from a minor tail vein bleed in fasted (8 h) *ob/ob* mice. Blood glucose was measured using a handheld glucometer (Bayer Contour, Leverkusen, Germany) prior ($t=0$) to administering a glucose challenge (18.8 mmol/kg/ bodyweight). Once administered, blood glucose was further analysed at 15, 30, 60, 90 and 120 min. Plasma insulin concentration was determined using insulin radioimmunoassay (described in 2.2.3).

Delayed glucose lowering effect In vivo

Hydrolysates that were identified as having an oral glucose lowering effect were then carried onto further investigation using delayed glucose tolerance test. This was investigated via oral compound administration during fasting, followed by glucose (18.8 mmol/L) administration either 4, 8 or 12 h later to identify if a lasting glucose lowering effect was evident from the hydrolysate of interest. The glucose-lowering dose of the hydrolysate were previously identified, animals were then fasted for 4 hours and then administered a dose of hydrolysate depending on its glucose lowering effect. Animals were given access to only water and at 4, 8

or 12 hour hours the animals were then given glucose (18.8 mmol/L) via oral gavage. Further experimentation procedures can be found at section 2.3.1.

Satiety and appetite study in 3 hour fed trained animals

Male Hsd:Ola T0 mice (8 weeks old) were obtained from Envigo, Blackthorn, UK. Mice were housed in an air conditioned room maintained within a narrow range of $22 \pm 2^{\circ}\text{C}$ with a 12 h light: 12 h dark cycle. Drinking water and standard rodent diet was freely available on arrival. All animal studies were performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

On arrival animals had access to food 24 hours per day for 1 week. This was reduced to 10 hours of food availability on week 2 with further reduction to 6 hours by week 3. Finally on week 4 and for the remaining duration of the animal satiety study, food availability was 3 hours per day from 10 am to 1 pm. Full experimental protocol can be found at section 2.3.4.

Statistical analysis

Data was expressed as mean \pm SEM with values compared using one-way analysis of variance (ANOVA) followed by post-hoc students T-test unless otherwise stated. Data between groups were considered statistically significant if $p \leq 0.05$. Data was plotted and analysed using Graphpad Prism 5.

5.3 Results

Fig 5.1 / 2 - Establishing the dose required to improve glycaemic parameters after oral administration of Boarfish protein hydrolysate and glucose

Groups of animals (n=8) were fasted for 12 h prior to an oral glucose challenge. Fasting blood glucose was established prior to administration of glucose and various concentrations of boarfish protein hydrolysate (BPH) ranging from 10 to 100 mg/kg/bw. Blood glucose concentration was subsequently measured at intervals over a 120 min period. Initially concentrations of BPH administered at 10 and 25 mg/kg/BW had no effect on glycaemic parameters (fig 5.1 A). Furthermore, an improvement was reported with concentrations of 50 mg/kg/bw and above. The 50 mg/kg/bw had reported a significant improvement in glycaemic parameter at 15 min ($p<0.05$), 30 min ($p<0.001$) and 60 min ($p<0.05$) versus the glucose only control (Fig 5.1 B). Interestingly, at higher administered doses, both 75 mg/kg/bw and 100 mg/kg/bw had only shown a significant glucose reduction at 30 min ($p<0.01$) over the 120 min time period (Fig 5.1 C). The SGID variant had no effect upon improving glycaemic control when administered compared to the glucose only control (fig 5.2).

Fig 5.3 - Establishing the dose required to improve glycaemic parameters after oral administration of Blue whiting protein hydrolysate (BWPH) and glucose

Initially concentrations of BWPH administered at 10 and 50 mg/kg/BW had no effect on glycaemic parameters (Fig 5.3 A / B). An improvement was reported however, with a concentration of 100 mg/kg/bw (Fig 5.3 C). The 100 mg/kg/bw had reported a significant improvement in glycaemic parameter at 90 min ($p<0.01$), and 120 min ($p<0.01$) versus the glucose only control.

Fig 5.4 - Establishing the dose required to improve glycaemic parameters after oral administration of *P.palmata* protein hydrolysate (PPPH) and glucose

An improvement was reported with concentrations of 50 mg/kg/bw and above. The 50 mg/kg/bw had reported a significant improvement in glycaemic parameter at 90 min ($p < 0.01$), and 120 min ($P < 0.001$) versus the glucose only control (Fig 5.4 B). At higher administered doses, 100 mg/kg/bw had shown an further improved glucose reduction at 60 min ($p < 0.01$), 90 min ($p < 0.001$) and 120 min ($P < 0.001$) versus the glucose only administered control (Fig 5.4 C).

Fig 5.5 - Establishing the dose required to improve glycaemic parameters after oral administration of *Salmon gelatine* protein hydrolysate (SGPH) and glucose

Concentrations of SGPH administered at 10 mg/kg/BW had no effect on glycaemic parameters (Fig 5.5 A). Furthermore, an improvement was reported with concentrations of 50 mg/kg/bw only (Fig 5.5 B) and not at 100 mg/kg/bw (Fig 5.5 C). The 50 mg/kg/bw had reported a significant improvement in glycaemic parameter at 30 min ($p < 0.05$), 60 min ($p < 0.05$), 90 min ($p < 0.01$) and 120 min ($p < 0.05$) versus the glucose only control. At higher administered dose, 100 mg/kg/bw no improvement in glycaemic parameters were noted versus the glucose only control, respectively (Fig 5.5 A – C).

Fig 5.6 - Establishing the dose required to improve glycaemic parameters after oral administration of *Salmon trimmings* protein hydrolysate (STPH) and glucose

A Concentration of STPH administered at 10 mg/kg/BW had no effect on glycaemic parameters (Fig 5.6 A). An improvement was reported with concentrations of 50 mg/kg/bw and 100 mg/kg/bw. The 50 mg/kg/bw had reported a significant improvement in glycaemic

parameter at 60 min ($p < 0.05$), 90 min ($p < 0.05$), and 120 min ($p < 0.05$) versus the glucose only control (Fig 5.6 B). At a higher administered dose of 100 mg/kg/bw glycaemic parameters were improved upon at the same time points with a modest improvement over 50 mg/kg at 90 min ($p < 0.01$) versus the glucose only control, respectively (Fig 5.6 C).

Fig 5.7 A - Establishing the mechanism of improved glycaemic parameters after oral administration of Boarfish protein hydrolysate (BPH) via circulating insulin concentration

Circulating plasma insulin levels were measured over the same time period of 120 min. When analysed via insulin area above the curve (AAC), BPH reported a significant total increase of circulating insulin levels ($p < 0.05$) versus the glucose only control (Fig 5.7 A).

Fig 5.7 B - Establishing the mechanism of improved glycaemic parameters after oral administration of *P.palmata* protein hydrolysate (PPPH) via circulating insulin concentration

When analysed via insulin area above the curve (AAC), PPPH reported a significant total increase of circulating insulin levels ($p < 0.05$) versus the glucose only control (Fig 5.7 B).

Fig 5.8 A - Establishing the mechanism of improved glycaemic parameters after oral administration of *Blue whiting* protein hydrolysate (BWPH) via circulating insulin concentration

When analysed via insulin area above the curve (AAC), BWPH reported no significant total increase of circulating insulin levels versus the glucose only control (Fig 5.8 A).

Fig 5.8 B - Establishing the mechanism of improved glycaemic parameters after oral administration of Salmon Trimmings protein hydrolysate (STPH) via circulating insulin concentration

When analysed via insulin area above the curve (AAC), STPH reported a significant total increase of circulating insulin levels ($p < 0.05$) versus the glucose only control (Fig 5.8 B).

Fig 5.9 A - Establishing the mechanism of improved glycaemic parameters after oral administration of Salmon gelatine protein hydrolysate (SGPH) via circulating insulin concentration

When analysed via insulin area above the curve (AAC), SGPH reported a elevated insulin trend however a non-significant increase versus the glucose only control (Fig 5.9 A).

Fig 5.10 A - Establishing the delayed glycaemic effect duration after oral administration of Salmon gelatine protein hydrolysate (SGPH) over a 12 hour period

An oral glucose challenge was carried out however; animals were administered SGPH at 4 h, 8 h or 12 h prior. Blood glucose levels were measures prior to glucose challenge (T=0 min) and at intervals to 120 min post glucose challenge. Administration of SGPH had no improvement in glycaemic parameters over the 120 min period post 8 and 12 h administration of SGPH, however an improvement at 15 min ($p < 0.05$) was noted 4 h post SGPH. There was a glucose trend reduction for both 4 h and 8 h which both returned an overall glucose reduction when analysed via area under the curve ($p < 0.05$) compared to the glucose only control (Fig 5.10 A).

Fig 5.10 B - Establishing the delayed glycaemic effect duration after oral administration of Salmon trimmings protein hydrolysate (STPH) over a 8 hour period

Administration of STPH had no improvement in glycaemic parameters over the 120 min period post 4 and 8 h administration of STPH. There was no glucose trend reduction for both 4 h and 8 h when analysed via area under the curve compared to the glucose only control (Fig 5.10 B).

5.11 A - Establishing the delayed glycaemic effect duration after oral administration of Boarfish protein hydrolysate (BPH) over a 8 hour period

Administration of BPH had no improvement in glycaemic parameters over the 120 min period post 8 h administration of BPH, however an improvement at 15 min ($p < 0.05$) was noted 4 h post BPH. There was a glucose trend reduction for 4 h which both returned an overall glucose reduction when analysed via area under the curve ($p < 0.05$) compared to the glucose only control (Fig 5.11 A).

5.4.11 B - Establishing the delayed glycaemic effect duration after oral administration of Blue whiting protein hydrolysate (BWPH) over a 8 hour period

An oral glucose challenge was carried out however; animals were administered SGPH at 4 h and 8 h prior. Blood glucose levels were measures prior to glucose challenge ($T=0$ min) and at intervals to 120 min post glucose challenge. Administration of BWPH had no improvement in glycaemic parameters over the 120 min period post 4 h or 8 h administration of BWPH.

5.12 B - Establishing the delayed glycaemic effect duration after oral administration of *P.palmata* protein hydrolysate (PPPH) over a 12 hour period

Administration of PPPH had no improvement in glycaemic parameters over the 120 min period post 4 h, 8 h or 12 h administration of PPPH versus glucose only treated control (Fig 5.12 B).

Fig 5.13 A / B– Investigating the reduction in food intake post IP administration of satiety promoting peptide hormones in animals trained to eat for 3 h per day

Animals trained to eat for 3 h/day were administered via IP injection, saline only, or saline with either Exendin-4 or CCK-8, both of which are known to be potent promoters of satiety causing a reduction of food intake. Food was weighed prior to availability and subsequently measured at 30 min intervals over a 180 min period. Both Exendin-4 and CCK-8 resulted in a significant reduction post-injection from 30 min through 180 min ($p < 0.001$, 30 min – 0.001, 180 min) versus the saline only control group (Fig 5.13 A/B).

Fig 5.13 C – Investigating the reduction in food intake post oral administration of P.palamata protein hydrolysate (PPPH) in animals trained to eat for 3 h per day

A reduction of food intake was only evident within the group administered 100 mg/kg bw PPPH which resulted in a significant reduction at 60 min through 120 min ($p < 0.05$ – $p < 0.01$) versus the saline only control (Fig 5.13 C).

Fig 5.13 D – Investigating the reduction in food intake post oral administration of Boarfish protein hydrolysate (BPH) in animals trained to eat for 3 h per day

No reduction of food intake was evident within the group administered BPH versus the saline only control (5.13 D).

Fig 5.14 A – Investigating the reduction in food intake post oral administration of Blue whiting protein hydrolysate (BWPH) in animals trained to eat for 3 h per day

No reduction of food intake was evident within the group administered BWPH versus the saline only control (Fig 5.14 A).

Fig 5.14 B – Investigating the reduction in food intake post oral administration of Salmon gelatine protein hydrolysate (SGPH) in animals trained to eat for 3 h per day

A reduction in food intake was only evident at 90 min ($p < 0.001$) after 50 mg/kg/bw whereas after 100 mg/kg/bw a significant reduction of food intake was evident from 90 min through 180 min ($p < 0.05$ – $p < 0.001$) versus saline control group (Fig 5.14 B).

5.14 C – Investigating the reduction in food intake post oral administration of Salmon trimmings protein hydrolysate (STPH) in animals trained to eat for 3 h per day

A reduction of food intake was evident within both groups administered STPH at 50 mg/kg/bw and 100 mg/kg/bw versus the saline only control. A reduction at both administered concentrations was reported from 30 min through 180 min ($p < 0.05$ – $p < 0.001$) versus saline control group (Fig 5.14 C).

Fig 5.1

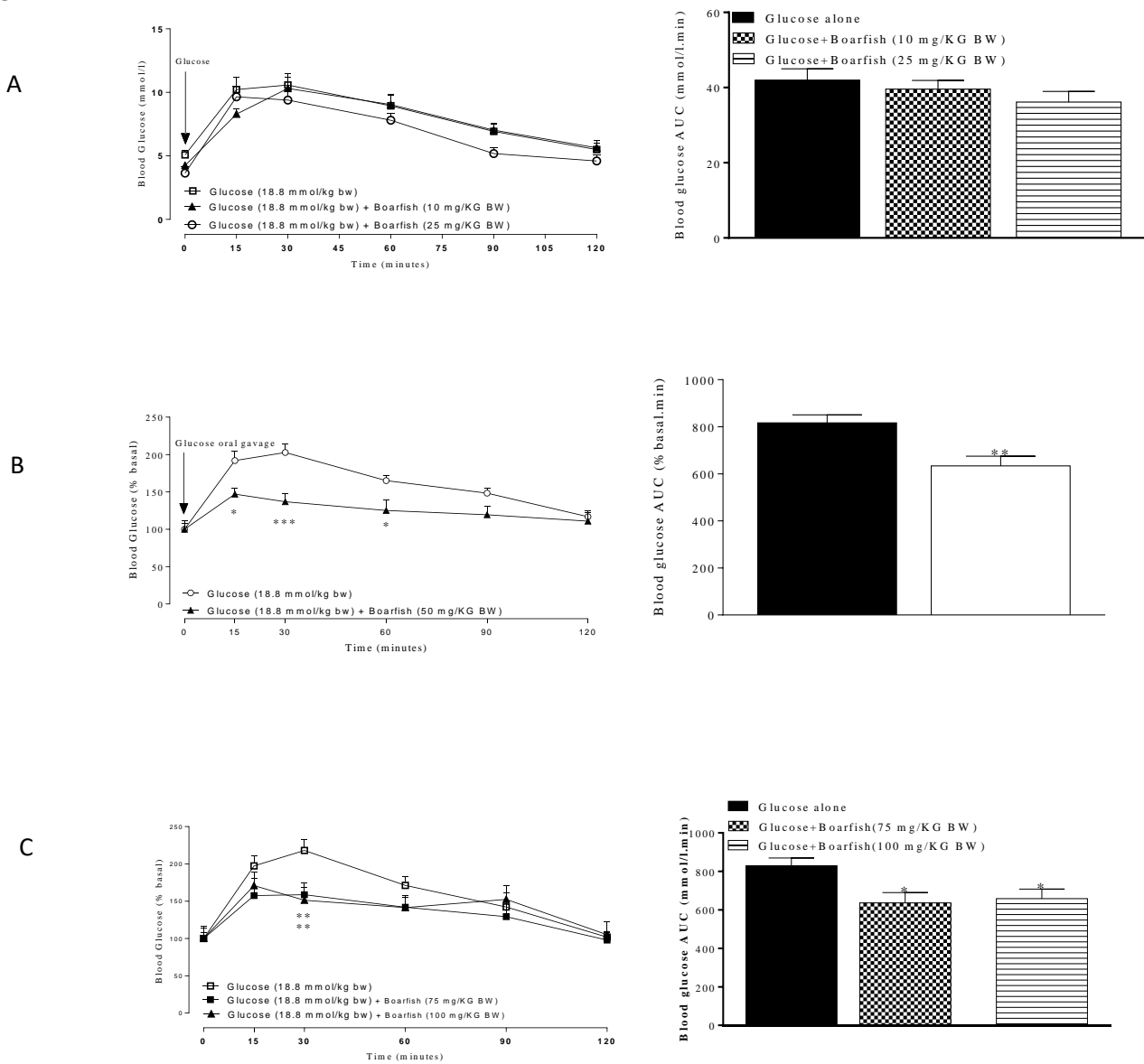


Fig 5.1. Acute actions of Boarfish hydrolysates on oral glucose tolerance in NIH Swiss mice. Glucose was measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; oral gavage) or in combination with extract (**Panel A** 10 / 25 mg/kg bw, **Panel B** 50mg/kg bw, **Panel C** 75-100 mg/kg body weight; oral gavage). Integrated panels (Right) show glucose AUC values for 0-120 min post-oral administration. Values are expressed as mean \pm S.E.M. for 8 mice. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to

Fig 5.2

D

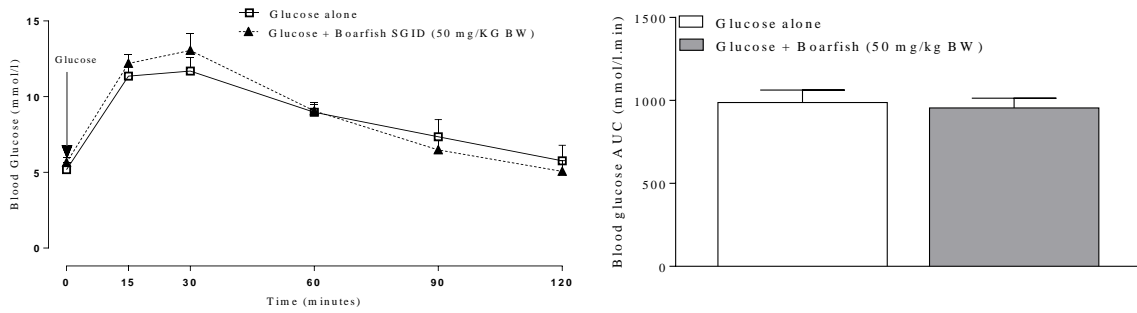


Fig 5.2. Acute actions of SGID Boarfish hydrolysate on acute oral glucose tolerance in NIH Swiss mice. Glucose was measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; oral gavage) or in combination with boarfish (**Panel A**) 50 mg/kg bw; oral gavage). Integrated panels (Right) show glucose AUC values for 0-120 min post-injection. Values are expressed as mean \pm S.E.M. for 8 mice. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to glucose alone.

Fig 5.3

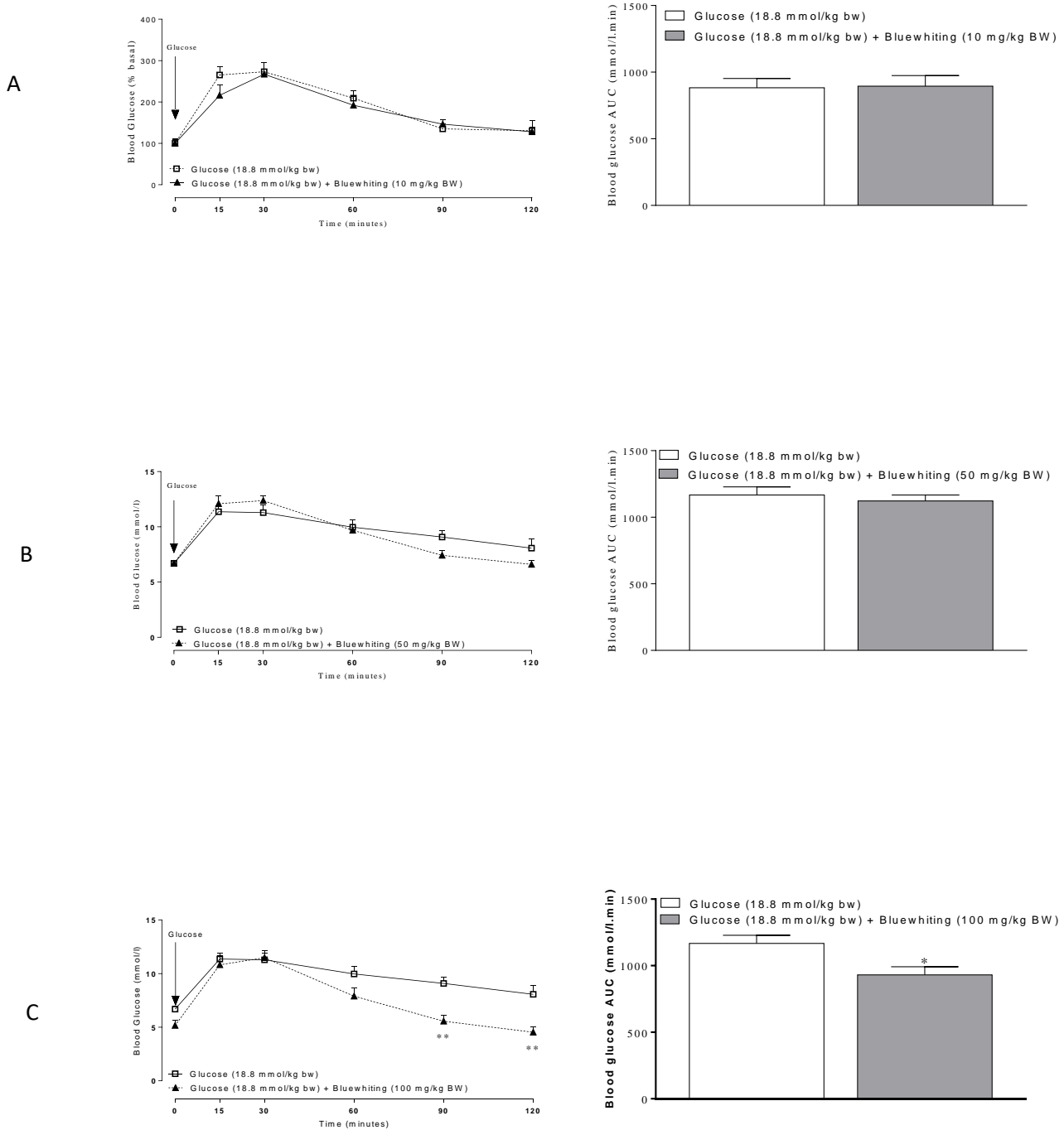
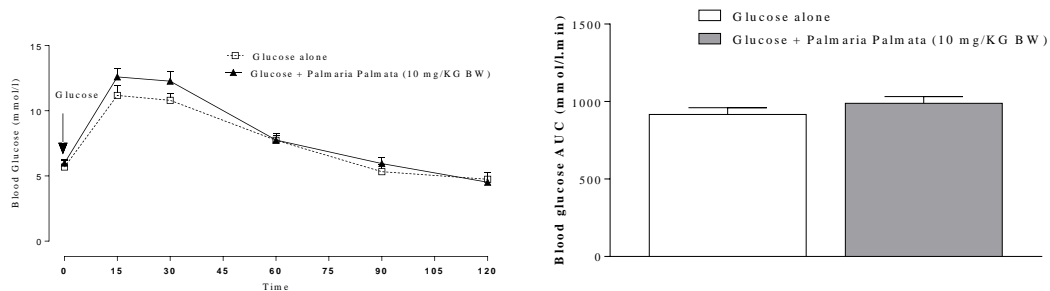


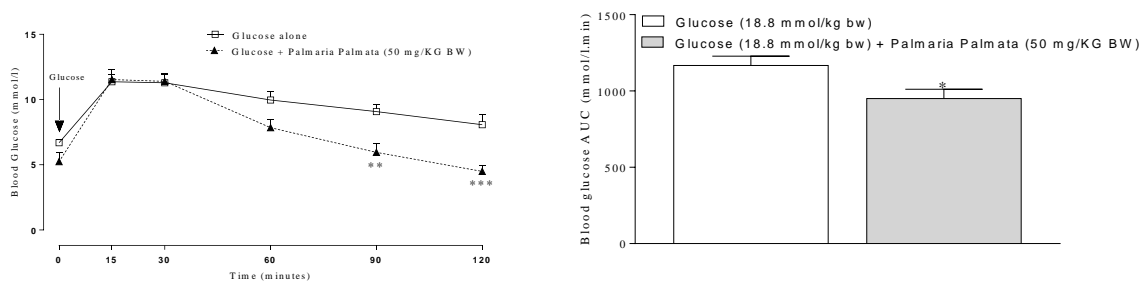
Fig 5.3. Acute actions of blue whitening hydrolysates on oral glucose tolerance in NIH Swiss mice. Glucose was measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; oral gavage) or in combination with extract (**Panel A** 10 mg/kg bw, **Panel B** 50mg/kg bw, **Panel C** 100 mg/kg body weight; oral gavage). Integrated panels (Right) show glucose AUC values for 0-120 min post-oral administration. Values are expressed as mean \pm S.E.M. for 8 mice. *p<0.05, **p<0.01 compared to glucose alone.

Fig 5.4

A



B



C

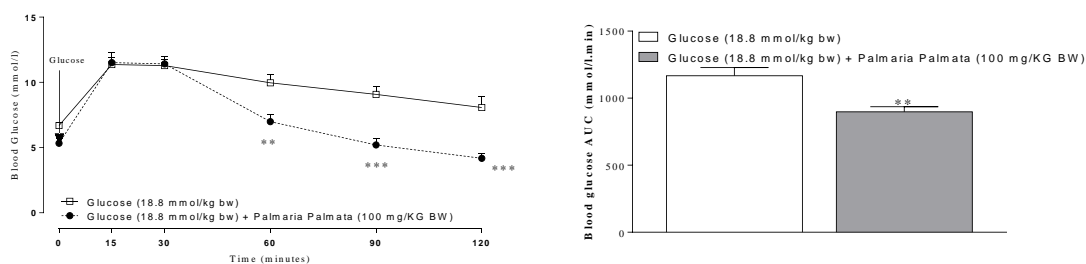


Fig 5.4. Acute actions of palmaria palmata hydrolysates on oral glucose tolerance in NIH Swiss mice. Glucose was measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; oral gavage) or in combination with extract (**Panel A** 10 mg/kg bw, **Panel B** 50mg/kg bw, **Panel C** 100 mg/kg body weight; oral gavage). Integrated panels (Right) show glucose AUC values for 0-120 min post-oral administration. Values are expressed as mean \pm S.E.M. for 8 mice. * $p < 0.05$, ** $p < 0.01$ compared to glucose alone.

Fig 5.5

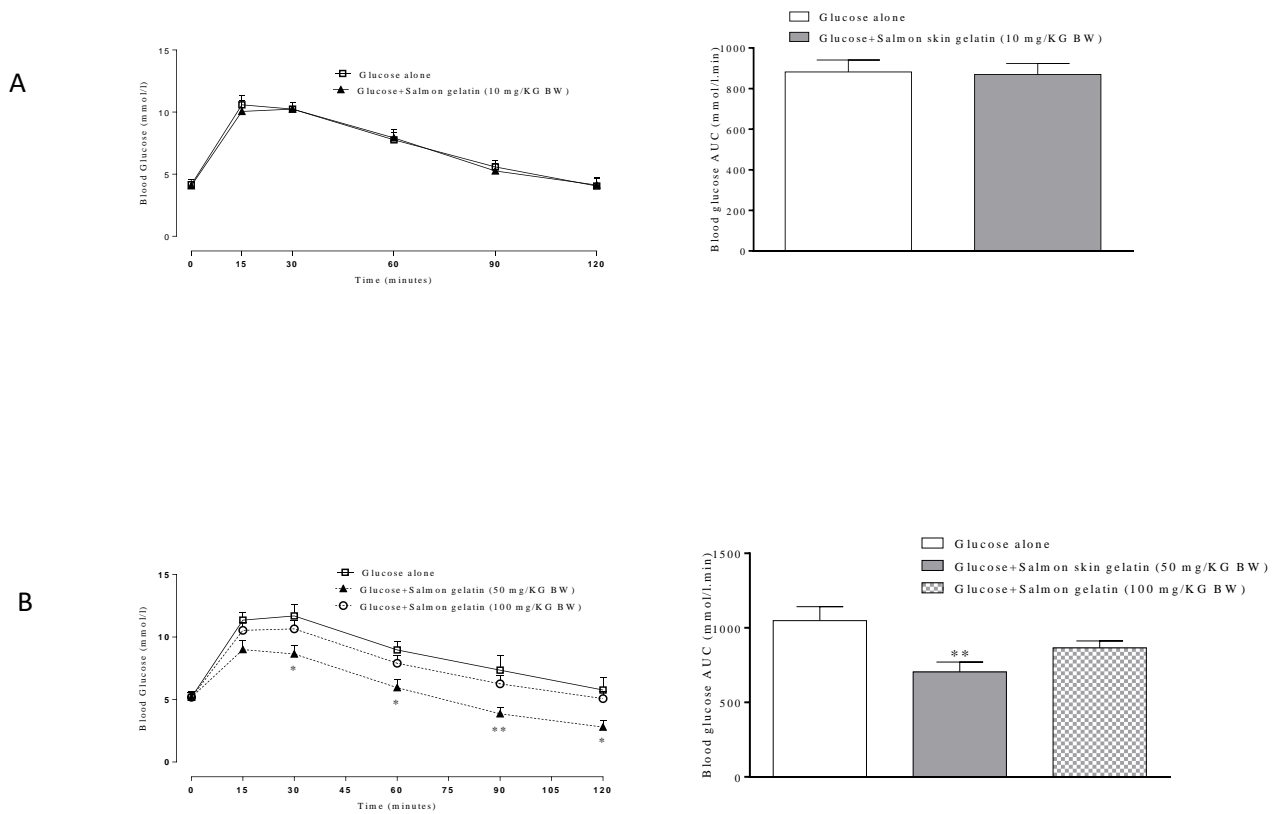


Fig 5.5. Acute actions of salmon gelatine hydrolysates on oral glucose tolerance in NIH Swiss mice. Glucose was measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; oral gavage) or in combination with extract (**Panel A** 10 mg/kg bw, **Panel B** 50 – 100 mg/kg bw; oral gavage). Integrated panels (Right) show glucose AUC values for 0-120 min post-injection. Values are expressed as mean \pm S.E.M. for 8 mice. * $p < 0.05$, ** $p < 0.01$ compared to glucose alone.

Fig 5.6

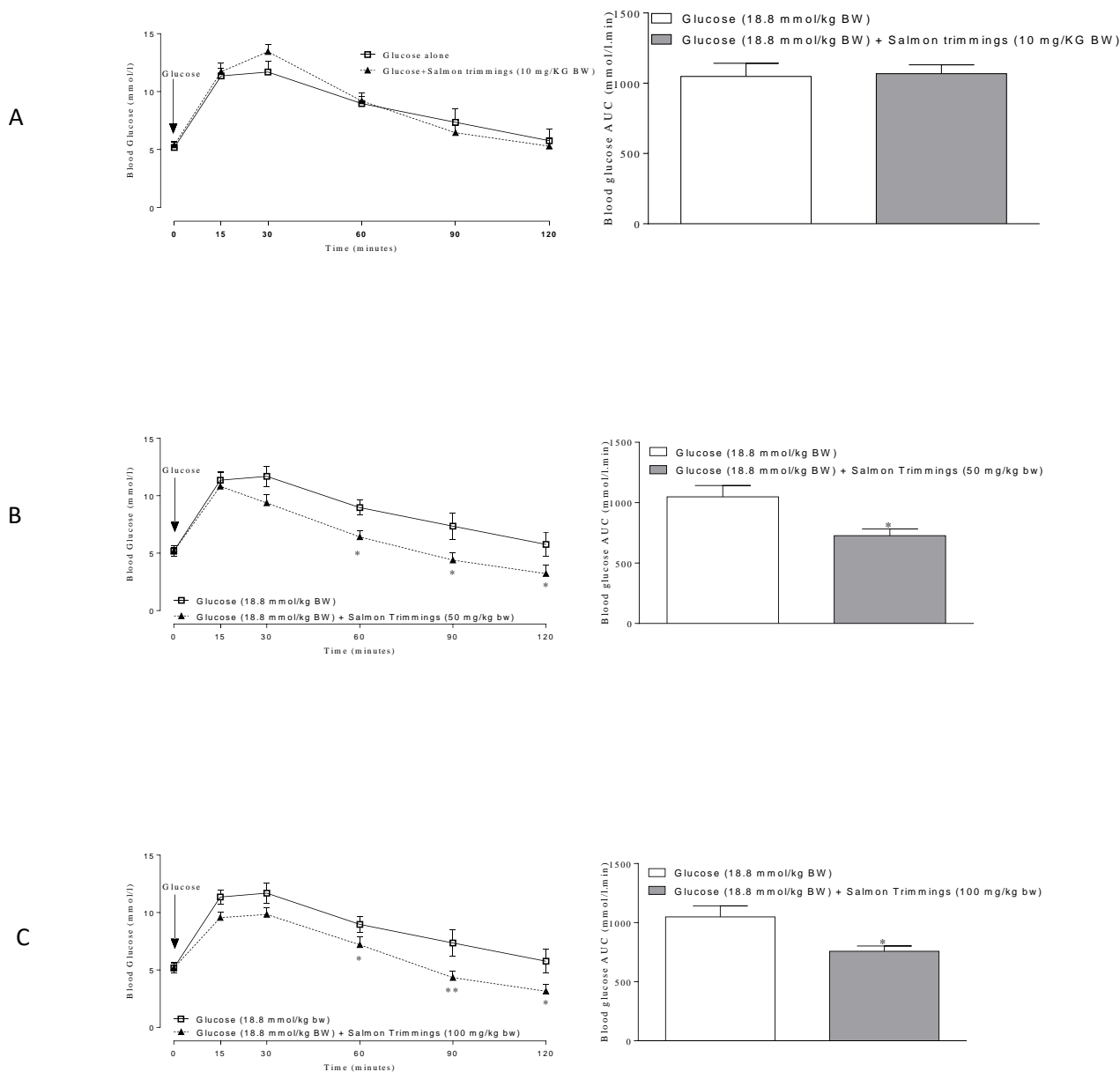


Fig 5.6. Acute actions of salmon trimming hydrolysates on oral glucose tolerance in NIH Swiss mice. Glucose was measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; oral gavage) or in combination with extract (**Panel A** 10 mg/kg bw, **Panel B** 50 mg/kg bw; **Panel C** 100 mg/kg bw oral gavage). Integrated panels (Right) show glucose AUC values for 0-120 min post-oral administration. Values are expressed as mean \pm S.E.M. for 8 mice. * $p < 0.05$, ** $p < 0.01$ compared to glucose alone.

Acute insulinotropic action of marine protein hydrolysates

Fig 5.7

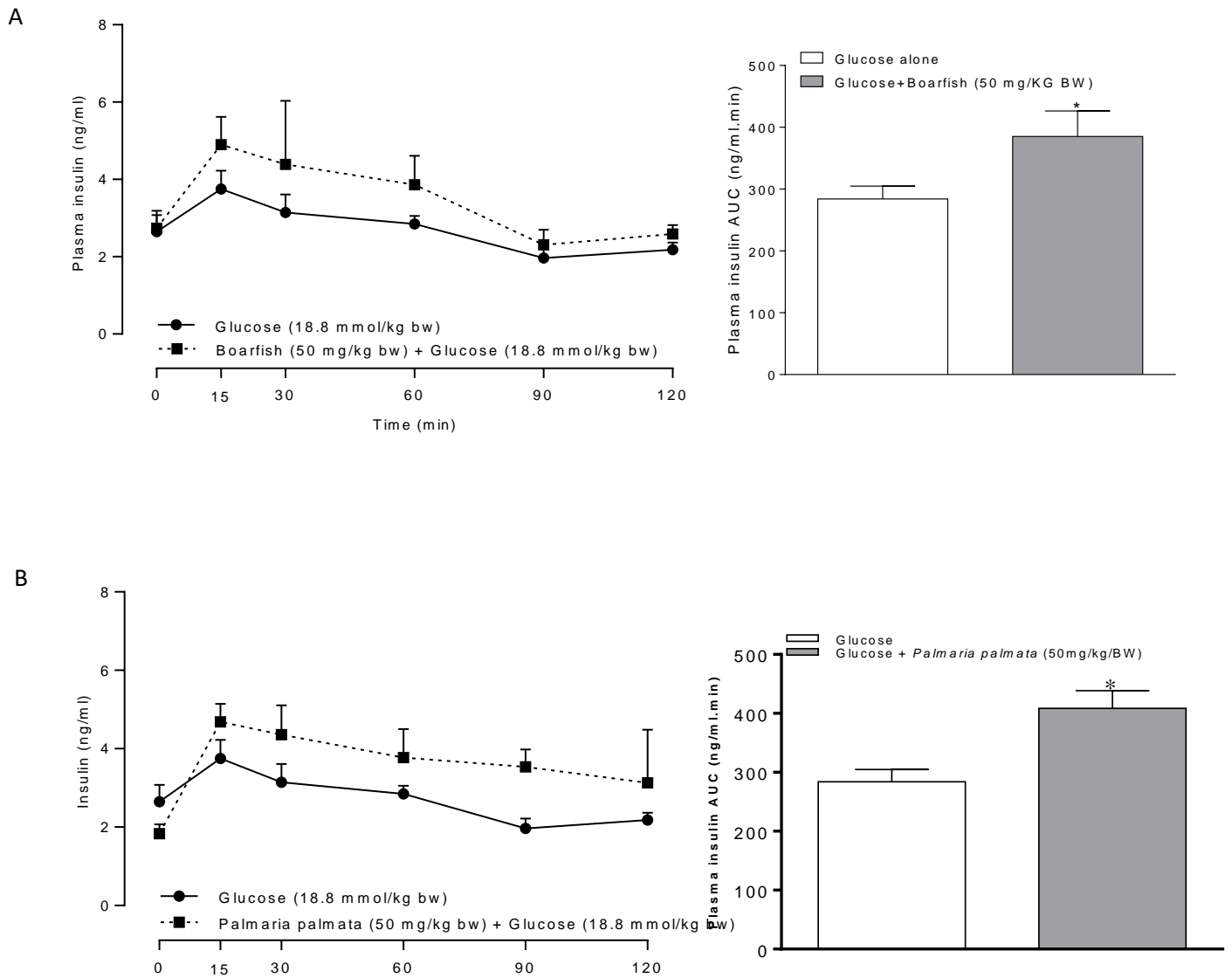


Fig 5.7. Acute insulinotropic action of marine hydrolysates generated using Alcalase/Flavourzyme combination, Boarfish (**Panel A**) and *Palmaria palmata* (**Panel B**) during oral glucose tolerance in NIH Swiss mice. Plasma insulin concentration was measured via RIA prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; oral gavage) or in combination with hydrolysates. Integrated panels (Right) show glucose AUC values for 0-120 min post-oral administration. Values are expressed as mean \pm S.E.M. for 8 mice. * $p < 0.05$, compared to glucose alone.

Fig 5.8

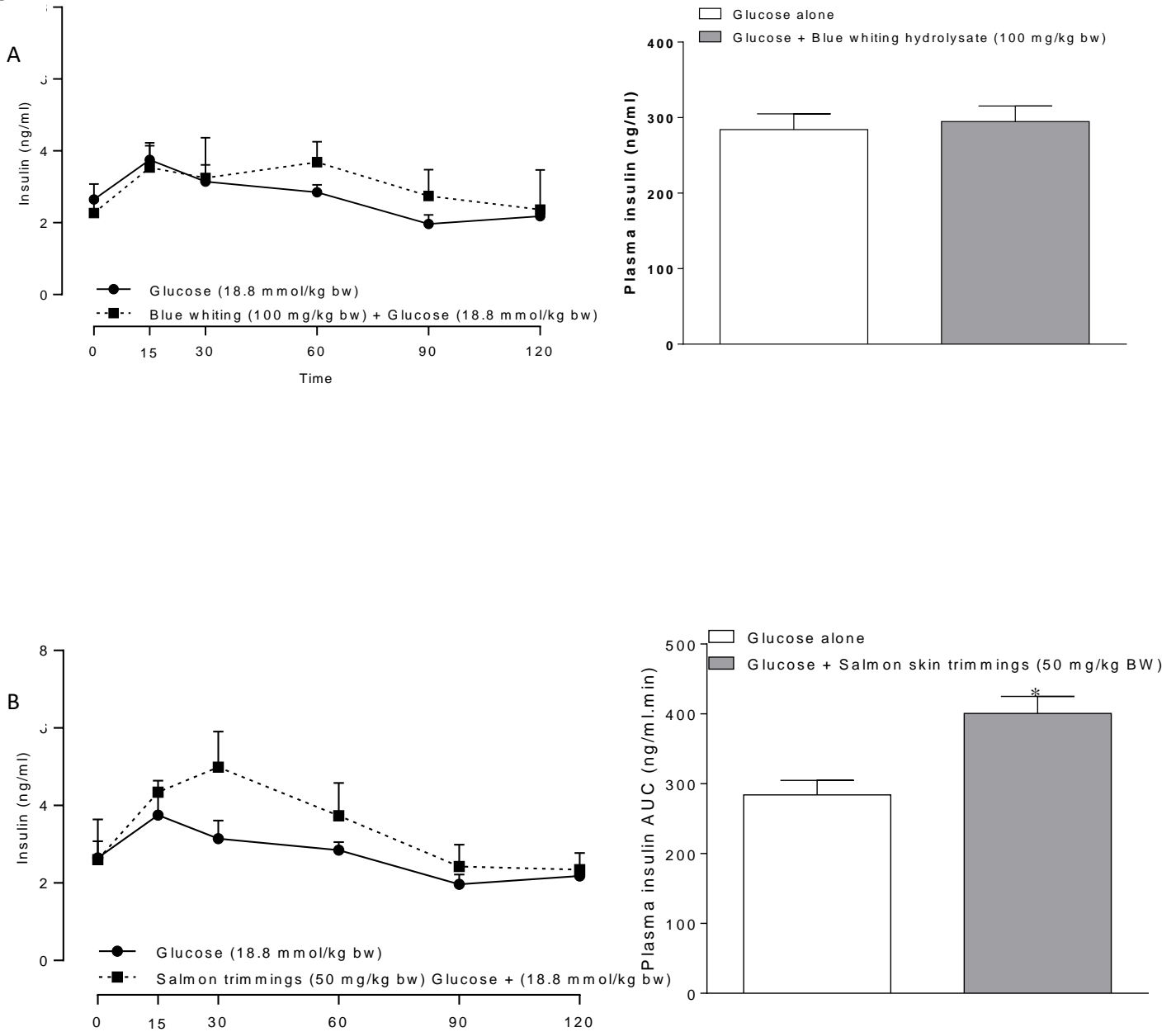


Fig 5.8. Acute insulintropic action of marine hydrolysates generated using Alcalase/Flavourzyme combination, Blue whiting (**Panel A**) and Salmon trimmings (**Panel B**) during oral glucose tolerance in NIH Swiss mice. Plasma insulin concentration was measured via RIA prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; oral gavage) or in combination with hydrolysates. Integrated panels (Right) show glucose AUC values for 0-120 min post-oral administration. Values are expressed as mean \pm S.E.M. for 8 mice. * $p < 0.05$, compared to glucose alone.

Fig 5.9

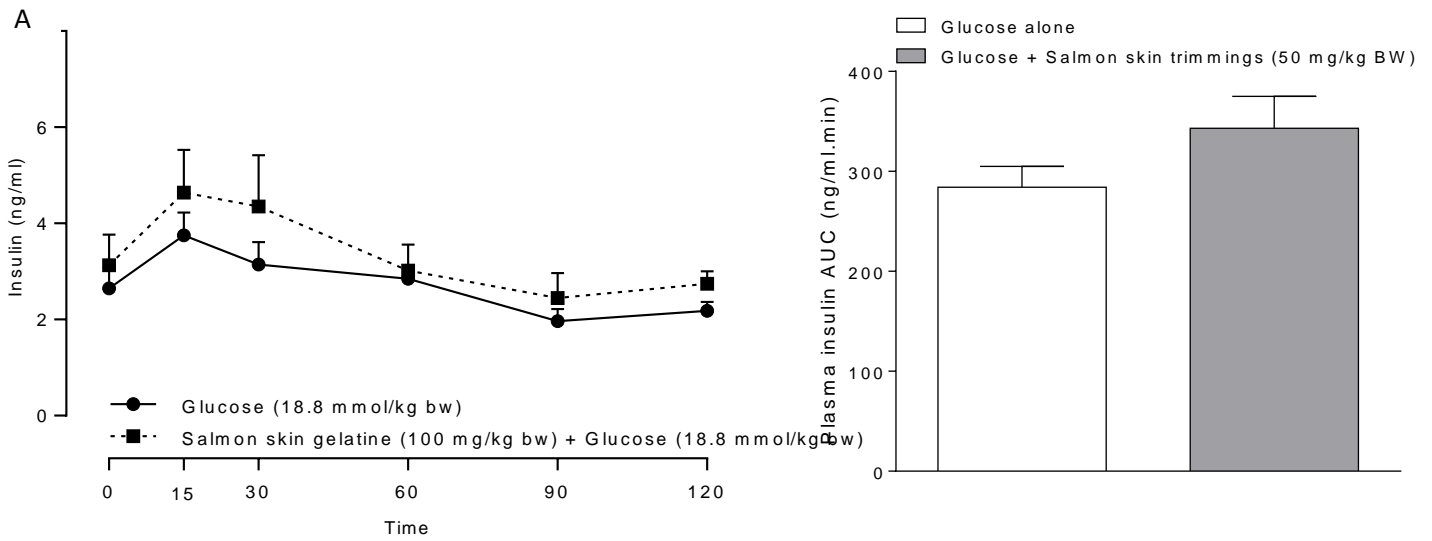


Fig 5.9. Acute insulintropic action of marine hydrolysates generated using Alcalase/Flavourzyme combination, Salmon skin gelatine (**Panel A**) during oral glucose tolerance in NIH Swiss mice. Plasma insulin concentration was measured via RIA prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; oral gavage) or in combination with hydrolysates. Integrated panels (Right) show glucose AUC values for 0-120 min post-oral administration. Values are expressed as mean \pm S.E.M. for 8 mice. * $p < 0.05$, compared to glucose alone.

Fig 5.10

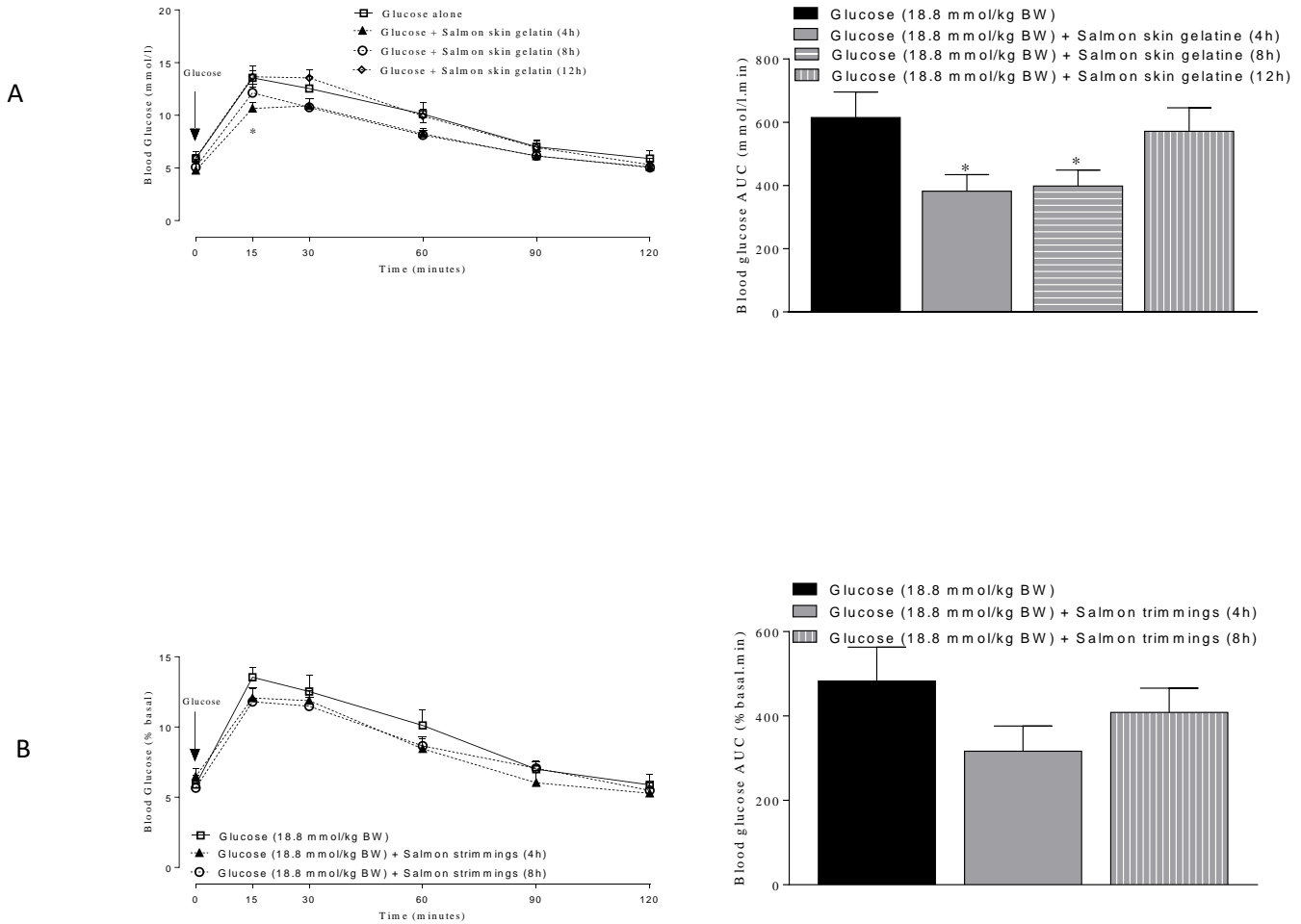


Fig 5.10. Delayed actions of salmon gelatine (**Panel A**) or salmon trimming (**Panel B**) hydrolysates on oral glucose tolerance in NIH Swiss mice. Hydrolysates were administered 4, 8 or 12 h prior to glucose. Blood glucose was then measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; oral gavage) or in combination with pre-administered extract (**Panel A** SSG 50 mg/kg bw 4, 8 & 12h prior to glucose **Panel B** ST 50 mg/kg bw 4 & 8 hours prior to glucose oral gavage). Integrated panels (Right) show glucose AUC values for 0-120 min post-oral administration. Values are expressed as mean \pm S.E.M. for 8 mice.

Fig 5.11

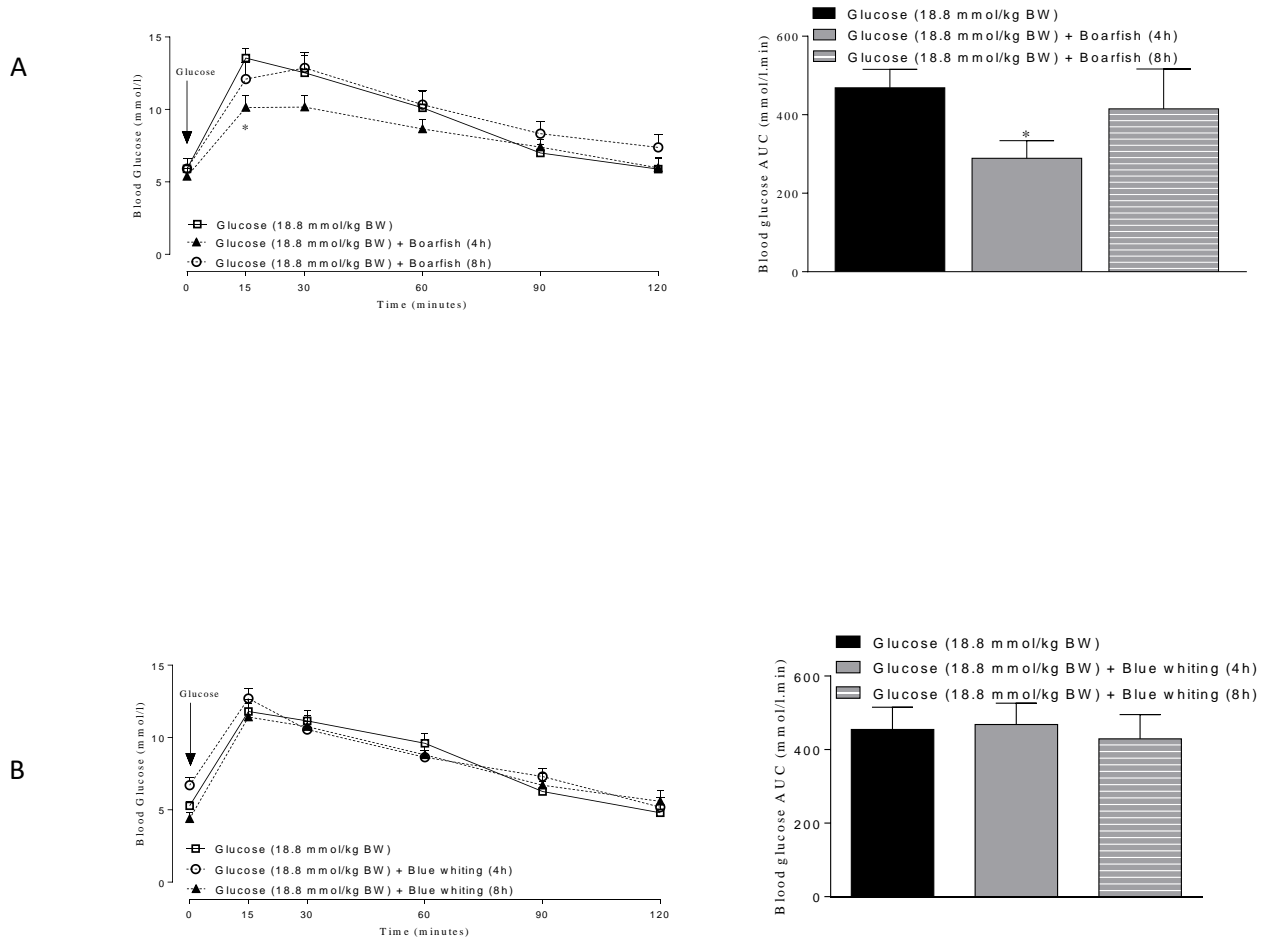


Fig 5.11. Delayed actions of boarfish (**Panel A**) or blue whiting (**Panel B**) hydrolysates on oral glucose tolerance in NIH Swiss mice. Hydrolysates were administered 4, 8 or 12 h prior to glucose. Blood glucose was then measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; oral gavage) or in combination with pre-administered extract (**Panel C** BF 50 mg/kg bw 4 & 8 H prior to glucose **Panel D** BW 50 mg/kg bw 4 & 8 hours prior to glucose oral gavage). Integrated panels (Right) show glucose AUC values for 0-120 min post-oral administration. Values are expressed as mean \pm S.E.M. for 8 mice.

Fig 5.12

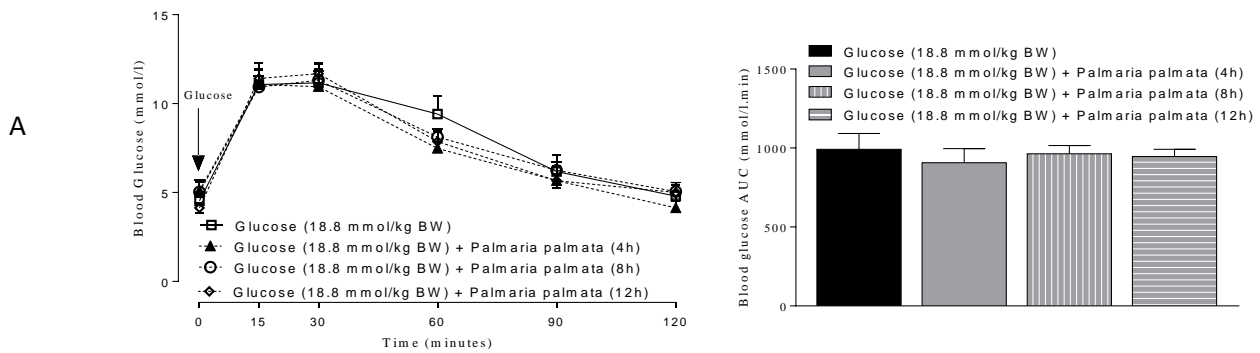


Fig 5.12. Delayed insulinotropic actions of palmaria palmata (**Panel A**) hydrolysates on oral glucose tolerance in NIH Swiss mice. Hydrolysates were administered 4, 8 or 12 h prior to glucose. Blood glucose was then measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; oral gavage) or in combination with pre-administered extract (**Panel E** 50 mg/kg bw 4, 8 & 12h prior to glucose oral gavage). Integrated panels (Right) show glucose AUC values for 0-120 min post-oral administration. Values are expressed as mean \pm S.E.M. for 8 mice. * $p < 0.05$ compared to glucose alone.

Fig 5.13

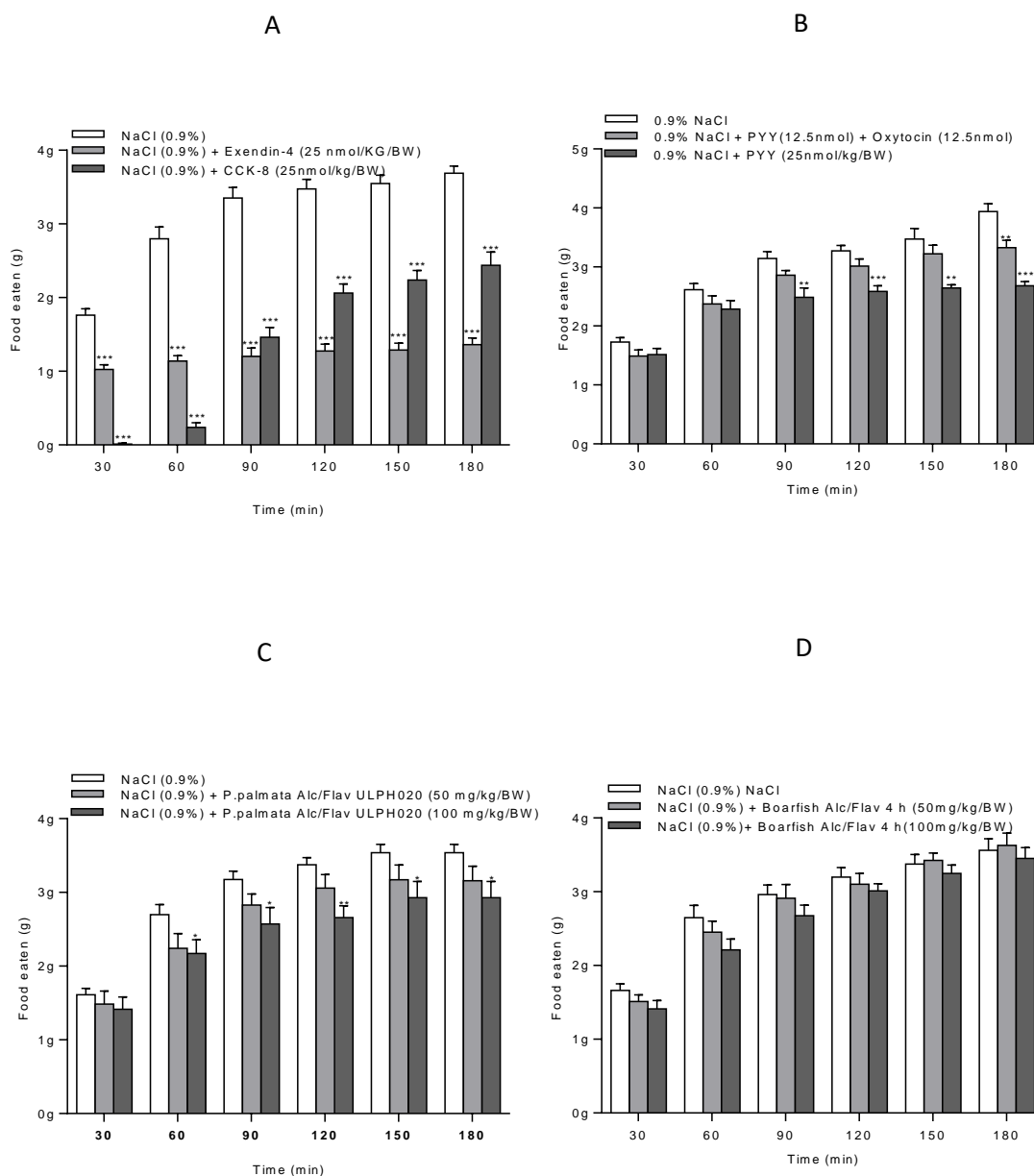


Fig 5.13. Food intake data for mice with restricted food availability (3 hours per day). Each group was given a compound (oral or IP). A weighed amount of food is given (T 0 min) and weighed every 30 minutes for 180 minutes. Marine Hydrolysates were administered orally, except panel A (IP) and Panel B (IP) with food eaten calculated accumulatively and comparative to non-treated saline control. Panel C represents oral palmaria palmata hydrolysates and panel D, boarfish hydrolysates effect on food intake. Values are expressed as mean \pm S.E.M. for 8 mice. * $p < 0.05$ ** $p < 0.01$ compared to glucose alone.

Fig 5.14

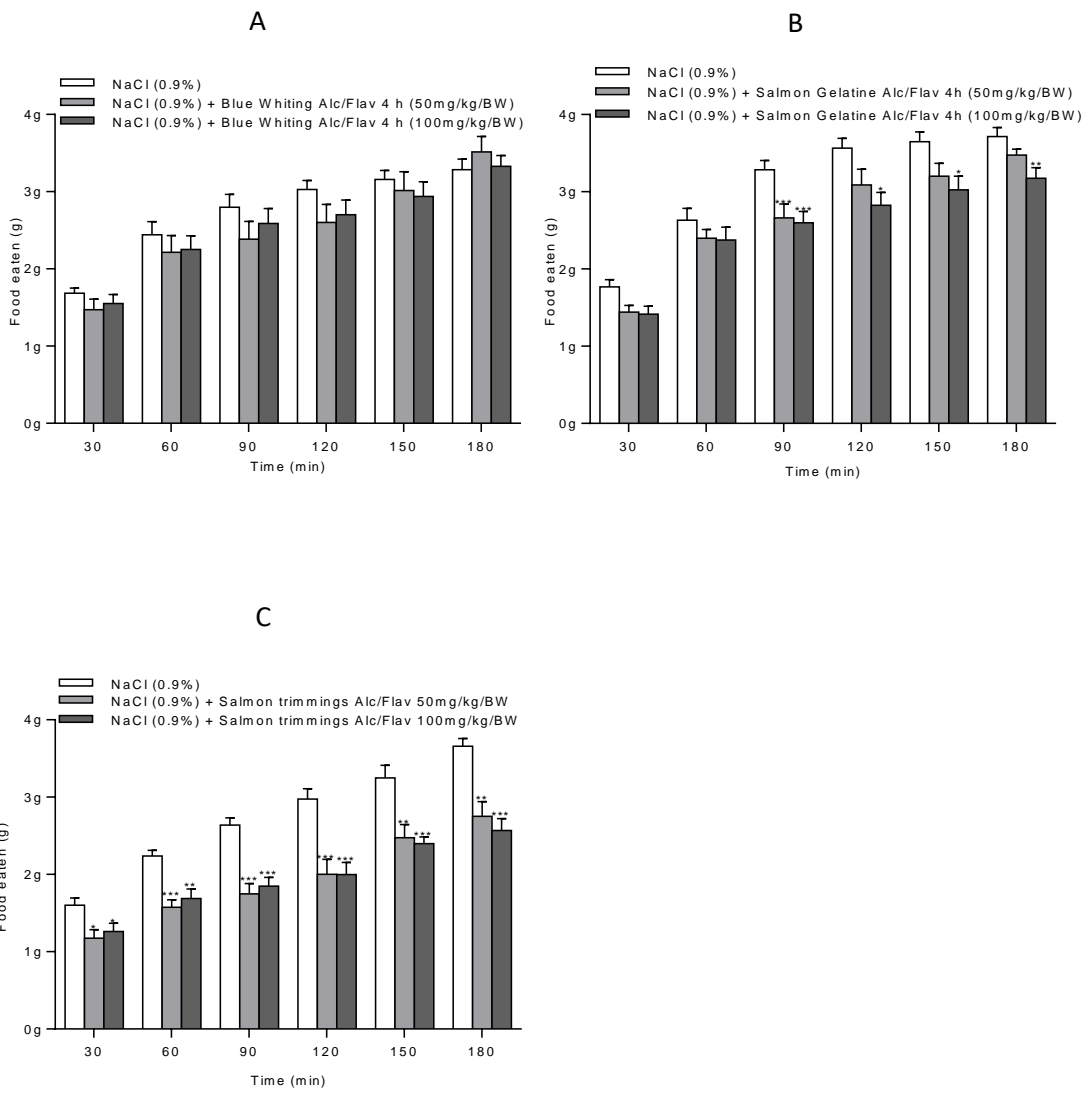


Fig 5.14. Food intake data for mice with restricted food availability (3 hours per day). Each group was given a compound (oral or IP). A weighed amount of food is given (T 0 min) and weighed every 30 minutes for 180 minutes. . Marine Hydrolysates were administered orally, (**Panel A**, Blue whiting, **Panel B**, Salmon gelatine, **Panel C**, salmon trimming) with food eaten calculated accumulatively and comparative to non-treated saline control. Values are expressed as mean \pm S.E.M. for 8 mice. * $p < 0.05$ ** $p < 0.01$ compared to glucose alone.

5.4 Discussion

Several decades of dietary research have highlighted that high protein diets can be successfully deployed to aid weight loss through improvements in body weight via humoral appetite regulation, however the overall outcome is neither consistent nor conclusive (Lepe *et al.* 2011). Protein loading prior to post-prandial food ingestion has shown improved satiety control without direct alteration of dietary macronutrients (Chambers *et al.*, 2015). Here, we studied whether co-ingestion of marine protein hydrolysates (MPH) from various biological sources showed improvements towards acute and delayed glucose tolerance including reducing food intake in trained mice. The oral glucose tolerance test still remains the gold standard for assess post-prandial glycaemic status. Upon challenging mice with glucose alone, or combined with protein hydrolysates including investigating the physiological dose required to promote improved glucose tolerance was assessed including detecting changes in plasma insulin concentration. Finally, analysis of food intake post-administration of MPH investigated which may hint at mechanisms that are insulin independent via satiety hormone or incretin hormone secretion mechanisms.

Within our own acute glucose tolerance tests, administration of hydrolysates at either 10 or 25 mg/kg/bw had no effect upon glycaemia. Interestingly, the dose required to improve post-prandial glucose tolerance was different throughout the biological sources. For example, Boarfish administered orally at 50 mg/kg/bw with glucose was the optimal dose required to promote a significant improvement in glycaemia over the 120 min observation duration. Furthermore, at higher doses of 75 and 100 mg/kg/bw, the positive effects within the later stages of observation at 60 min were lost. This may be due to the nature of protein source, still within a rather crude state including unwanted compounds, which may affect the outcome such as free fatty acids or triglycerides that were not removed in total during the delipidating process. In order to assess if the bioactivity and glucose lowering effect was derived from peptides or amino acids, the boarfish hydrolysate was further subjected to lab based simulation gastrointestinal digestion (4 h) using pepsin and trypsin enzymes.

After administration of the SGID boarfish protein hydrolysate at the same dose that had promoted the most potent glucose tolerance improvement, the glucose lowering effect was lost. This had indicated the effects upon insulin dependent of independent mechanisms were peptide derived, as the SGID variant would have contained a significantly higher proportion of its nitrogen source as amino acids rather than peptides. The optimal dose required for boarfish, salmon and algal hydrolysates was 50 mg/kg/bw and while further glycaemic improvements were seen with 100 mg/kg, this equates to 7 g for human consumption and may present issues around food incorporation, especially within western food stuffs. With this noted, blue whiting required the highest dose investigated to promote improve glucose tolerance within mice.

Protein intake has significant effect on insulin and glucagon, including liberated amino acids, specifically branch chain amino acids Val, Ile and Leu being potent predictors for insulin and glucagon response (Yoon, 2016; Chartrand *et al*, 2017). This study focused on peptides derived from marine protein after hydrolysis using proteolytic enzymes yielding an abundance of small molecular weight peptides. Recent review outcomes have showed advantages of high protein intake that is consistent with weight loss and lowered fat mass via humoral improvements (Pesta & Samuel, 2014), however, studies focusing on novel marine sources of protein hydrolysates and metabolic changes relating to post prandial glucose regulation and satiety are limited. The insulinotropic actions of orally administered hydrolysates within this study returned interesting results. The analysis was suggestive post-analysis of the lowest dose required to promote improved glucose tolerance of increased circulating plasma insulin however, this was only deemed significant when analysed via insulin area above the curve (AAC). Furthermore, blue whiting hydrolysate had not reported any effects upon increase insulin secretion versus the glucose only administered control group. This is suggestive of potentially non-insulinotropic mechanisms involved; however, without further investigation the effect upon glycaemia at present is speculative.

Finally, within this study investigating the effect upon food intake in trained animals was assessed. Satiety studies focus primarily on food-derived protein or supplemental dairy or plant based protein, showing lesser interest to liberated protein by-products in the form of peptides (Bendtsen *et al*, 2013). Nowadays however, the bioactive screening of novel proteins and peptides is one of the most exciting therapeutic directions aimed at tackling some of the most prevalent diseases within society (Giri & Ohshima. 2012; Jo *et al*, 2017). To date the evidence that oral intake of peptides and exogenous biopeptides can stimulate gluco-regulatory and satiating effect present a consistent relationship (Steinert *et al*, 2009). This link between satiety and improved glucose regulation is due to the diverse mechanisms that are displayed when a protein is digested, liberating small molecular weight peptides (SMWP) and amino acids. An increasing number of fish protein hydrolysates and derived peptides to date have exposed a range of anti-diabetic activity with improved glucose utilization in vivo promoting hyperglycaemic management however the precise mechanisms underlying these action are still to be determined (Zhu *et al*, 2017; Saleh *et al*, 2018).

Throughout this investigation, not all glucose lowering doses of marine hydrolysates translated into a reduction in food intake suggesting different insulin dependent and independent pathways being activated dependant on the peptide fragments generated via hydrolysis. Blue whiting hydrolysate generated promoted improved glucose excursion, however, only after 100 mg/kg oral administration, whereas the same dose had no effect on food intake. Furthermore, studies investigating the satiating effect of blue whiting hydrolysates have reported beneficial effects of 1.4 and 2.8 g increasing CCK and GLP-1 secretion in healthy volunteers (Nobile *et al*, 2016). Throughout our food intake investigation post-oral administration of hydrolysates at 50 or 100 mg/kg/bw, the lowest quantity of hydrolysate needed to promote a reduction in food intake was 100 mg/kg/bw. Furthermore, this was only evident after administration of the algal and salmon gelatine hydrolysate, however interestingly the salmon trimmings hydrolysate was equally potent at both 50 and 100 mg/kg displaying the most promising effects towards reducing food intake.

We hypothesize the mechanism of which food intake was reduced is primarily intestinal derived. Interaction of small marine peptides with large complex molecules have identified peptides from marine biomass, *palmaria palmata* (Dulse) which have also presented dipeptidyl-peptidase-IV (DPP4) inhibitory effect *in vitro* (Harnedy *et al*, 2015). Peptides derived and sequenced from the crude protein hydrolysate ranging from 4 to 11 amino acids long, presented DPP4 IC₅₀ inhibition at concentrations ranges of 43 - 139 µM, with 3 short protein fragments in particular showing promising activity, namely, Ile-Leu-Ala-Pro, Leu-Leu-Ala-Pro and Met-Ala-Gly-Val-Asp-His-Ile. Small peptides play advantageous metabolic roles versus their intact protein counterpart. Generally, the acute effect of a product leading to amelioration of post-prandial spikes in blood glucose or reduction in overall food intake is considered an advantageous approach to obesity and diabetes management.

Work involving multifaceted effects of marine protein hydrolysates acutely using *in vitro* and *in vivo* investigation has been promising (Harnedy *et al*, 2018). The aforementioned study had shown modest GLP-1 secretion from cultured murine enteroendocrine cells; however the SGID variant of blue whiting presented increased bioactivity regarding GLP-1 secretion over the non-SGID version of which was investigated within. Furthermore, this may explain the rationale towards requiring a larger administered dose of blue whiting while other hydrolysates such as boarfish and salmon were more promising at lower doses, however post-oral investigation of circulating plasma GLP-1 concentrations are needed.

Post-prandial incretin secretion and satiety hormone biomarkers were not established within this study however is warranted in future work, especially post-administration of salmon protein hydrolysate. This confirmed that the protein source and method used to generate the hydrolysate and liberate peptides fragments are the most important factors when producing a product with bioactivity after oral intake. Understanding the prolonged effects of protein and hydrolysate intake is widely studied. Research suggests that pre-loading protein may further enhance post-prandial glucose

homeostasis or perceived hunger with subsequent meals after the pre-loading stage however, some suggest that the satiety response is independent to CCK, GLP-1 and PYY secretion (Oesch *et al*, 2005).

The work presented within agrees with concerns and questions raised over protein and protein by-products that may promote therapeutic benefits for the treatment of obesity and type 2 diabetes. Firstly, the source of protein may be a major factor in producing a bioactive product and secondly the hydrolysis method used to liberate the bioactive components such as small molecular weight peptides may directly dictate the bioactivity. Furthermore, the *in vitro* activity does not always translate to *in-vivo*, seen with work published on blue whiting hydrolysates of which promote dose dependent secretion of insulin in BRIN-BD11 cells and GLP-1 secretion in GLUTag cells (Harnedy *et al*, 2018); however, no satiating effect was displayed when administered orally to mice.

Surprisingly with protein sources, such as salmon skin gelatine and trimmings, *in vitro* secretory activity was not as potent as other sources such as *P.palmata* or boarfish hydrolysates seen in cultured cells within previous chapters while salmon hydrolysates continued to prove very effective at improving glucose tolerance and promoting a potent reduction of food intake *in vivo*. We conclude that more importantly the hydrolysis method is most likely a vital step, with hydrolysates that are abundant in peptides less than 2kDa as this yield a higher concentration of peptides and free amino acids. This approach to glucoregulatory and effect on appetite was limited due to the full understanding of mechanisms behind the effects seen; however, with the insulinotropic and satiating effect displayed we can confirm that oral intake of hydrolysed marine protein has pleotropic actions within the gut or internal systems.

Chapter 6

Investigating efficacy of long-term twice-daily oral intake of Boarfish and Salmon skin gelatine protein hydrolysates in genetically-induced obese-diabetic (*Ob/Ob*) mice

6.1 – Introduction

Dietary advice for obese individuals with type 2 diabetes (T2D) recommend reducing foodstuffs containing refined carbohydrates and saturated fats as well as increasing fibrous foods may be useful in disease management (Asif, 2014; Gray, 2015). Overnutrition is currently the leading cause of type 2 diabetes globally with serious impacts on quality of life and life expectancy (Gupta *et al*, 2012). Type 2 diabetes, the most common endocrine disorder is expected to affect over 640 million people by 2040, and represents 100% rise from today's diagnosed rates (Marín-Peñalver *et al*, 2016). Once an issue faced primarily in western countries, it is now an environmental disaster of epidemic proportions. Weight gain is now regrettably a primary reality globally with ready access to highly processed, low cost convenience foods that are calorie dense (Mozaffarian *et al*, 2011). Weight loss approaches and maintenance of weight loss needs major and modern revitalization in order to improve weight associated disease outcomes (Champagne *et al*, 2012).

Overweight and obese individuals may present with unsuccessful weight loss attempts accompanied by compensatory homeostatic glucose regulation prior to the diagnosis of T2D directly related to over-nutrition (Chui *et al*, 2010). In those with T2D, hyperglycaemia, insulin resistance, hyperinsulinaemia and elevated glycated haemoglobin A1c (HbA_{1c}) are invaluable diagnostic biomarkers which allows strategic management using an arsenal of hypoglycaemic agents (Meigs, 2009; Lorenzati, *et al*, 2010). Dietary modification mentioned, including an increase in physical activity allow individuals to successfully manage and potentially prevent further progression of T2D (Sigal *et al*, 2006). Furthermore, with this noted, weight loss plateau and failure to adhere to the classical diet and lifestyle management approaches unfortunately are very often surpassed in favour of pharmacological treatment (Gibson & Sainsbury, 2017). In the past several decades alone, the effective use of metformin has been joined by eight classes of highly specific glucoregulatory drugs which aim to promote normoglycemia (Abbatecola *et al*, 2008; Zhang & Moller, 2000). These modern

pharmacological agents have diverse interaction with glucose homeostatic systems. Advances using Gliptins, known as DPP-4 inhibitors, prolong post-prandial incretin hormone action of GLP-1 and GIP, both potent glucose-dependant insulinotropic hormones with advantageous effects towards satiety and gastric emptying (Ahren, 2007; Pathak & Bridgeman, 2010). Improving upon endogenous hormones using modified incretin mimetics via injectable GLP-1 analogues act upon endogenous hormone pathways and receptors with increased half-lives and resistance to endogenous DPP-4 proteolytic digestion and are quickly becoming a viable second choice option for type 2 diabetes treatment, especially within the elderly due to their safety profile (Gupta, 2013; NICE NG28 2015, McBrayer & Tal-Gan, 2017;). Finally, in keeping with the gastrointestinal theme, alpha-glucosidase inhibitors (α -GIs) reduce post-prandial carbohydrate degradation within the intestine reducing glucose excursion from exogenous food polymers, inevitably lowering the post-prandial elevation of glucose (Van De Laar *et al*, 2005; Van De Laar, 2008).

There are many viable approaches to treating hyperglycaemia, insulin resistance and T2DM via the use of oral or injectable hypoglycaemic agents (Evans *et al* 2016); the few approaches listed above are of particular interest due to their post-prandial potency. Recently, several exciting approaches for attempting to exploit hormonal pathways via food derived biomolecules that exert direct or indirect interaction with satiety centres or improvement of gastrointestinal transitioning and signalling have become increasingly popular, however these have bioavailability limitations (Rekha & Sharma, 2013). Current evidence investigating total fibre or cereal fibre being inversely associated with the risk of developing type 2 diabetes has made its way into dietary recommendations for obese or pre- or post T2D. Rather than focus on bioavailability and absorption of biomolecules, studies have suggested that a high intake of fibre is associated with a 33% reduction of T2DM over those with lower fibre intake (The InterAct Consortium, 2015). The rationale being that both soluble and insoluble fibre components present effects for delaying the rate of which carbohydrate polymers are liberated into their monosaccharide forms prior to absorption at the intestinal mucosa membrane. Epidemiological

and investigatory work have shown that food derived or supplemented fibre improves fasting blood glucose concentrations and improves glycated haemoglobin A1c (HbA1c) (Post *et al*, 2012).

Nutritional recommendations such as reduction of overall carbohydrate and fat intake help support approaches to nutritional management of hyperglycaemia (Carreriro *et al*, 2016). Furthermore, newer approaches to obesity management are now being explored with high protein intake becoming increasingly popular with potential for offsetting other macronutrient intake by improving satiety. Dietary proteins are vital modulators of glucose metabolism, however, only a limited number of studies to date have attempted to unravel long-term protein intake and effect on satiety and glycaemia with renal function usually a key aim of investigation (Kamper & Strandgaard, 2017). Nevertheless, protein in maintenance of energy balance has considerable support however; several variables arise at present, such as the protein source, type and quantity required (Griffioen-Roose *et al*, 2012). Furthermore, short-term trials investigating various macronutrient loading upon glycaemia favoured high protein over high carbohydrate and thus have been suggestive for a viable nutritional approach for T2D prevention (Nuttal *et al*, 1984). Additionally, many studies up until now focus primarily on food derived protein which leaves many unanswered questions for other sources of protein and in particular, dietary supplemented protein or hydrolysed protein products and amino acids.

This present study aims to investigate bioactive protein by-products generated using enzymatic hydrolysis. Bioactive peptides are molecules derived from the intact proteins and consist of amino acid chains linked together by peptide bonds. In nature free-living peptides are known, but the overwhelming majority are contained within the protein structure (Herbst *et al*, 2004; Conlon & Mechkarska, 2014; Sarbon, 2017). Hydrolysis of protein yields an abundant source of small molecular weight peptides, which can be further characterised for bioactivity using a range of *in vitro* or *in vivo* investigation. Small molecular weight peptides generated using enzymatic hydrolysis are now of particular interest, as they may present further resistance to gastrointestinal degradation and interact

with systems that control metabolism, satiety, including interaction with large endogenous proteases that are of pharmacological relevance for hypertension, obesity and diabetes treatment (Hayes & Tiwara, 2015; Patil *et al*, 2015). There are many examples of biologically active food proteins however; evidence now supports that liberation of small molecular weight peptides (SMWP's) may presents advantageous biological effects over the parent proteins (Kitts & Weiler, 2003).

A study investigating DPP-4 inhibitory properties of peptides derived from protein extracted from an algal source (*Palmaria palmata*) after enzymatic hydrolysis. The peptides were generated using enzymatic digestion and fractioned using solid phase extraction (SPE) combined with semi-preparative high pressure liquid chromatography (HPLC). In total, 13 peptides were identified; three novel DPP-4 inhibitors returned IC₅₀ values in the range of 42 – 159 µM. These peptides, varying from four to seven amino acids in length liberated from parent protein molecule may have potential in the management or prevention of T2D as part of a functional food approach (Harnedy *et al*, 2015).

Protein, peptides and amino acids can regulate glycaemic control via a wide range of internal signalling mechanisms dependent on the primary sequence of the peptides or amino acids generated following digestion (Harnedy & Fitzgerald, 2011). Hydrolysates deployed within the present study were screened initially using acute *in vitro* and *in vivo* small rodent models to assess which enzymatic preparation presented promising effects prior to long-term administration in a diabetic mouse model. Other work investigating acute administration of marine protein hydrolysates generated using Alcalase and Flavourzyme in combination (2.4L/500L) from blue whiting (*Micromesistius poutassou*) presented potent and promising *in vitro* and *in vivo* data. The hydrolysate returned a DPP-4 IC₅₀ value of 1.28 mg/ml, including dose-dependent insulin secretion from cultured BRIN-BD11 cells ranging from 0.039 to 2.5 mg/ml (Harnedy *et al*, 2018). A limitation for any nitrogen containing biomolecule is the ability to survive in the harsh proteolytic conditions of the gastrointestinal (GI) environment and something that researchers must acknowledge (Renukuntla *et al*, 2013). Interestingly, laboratory based simulated gastrointestinal digestion (SGID) of the already hydrolysed protein yielded

comparable or improved insulinotropic actions in culture cells including improved glucose tolerance in mice following post-prandial insulinotropic actions (Harnedy *et al*, 2018b). Studies investigating long-term administration of marine protein hydrolysates have similarly returned anti-diabetic potential. Using a cohort of Chinese patients with T2D, a total of 100 diabetic patients and 50 health controls were recruited. The diabetic subjects received 13 g of marine collagen peptides for 1.5 or 3 months daily, and subsequently returned reduced levels of fasting blood glucose, glycated haemoglobin (A1c), improved fasting insulin, lipid parameters including lowered triglycerides and low-density lipoprotein and importantly increased levels of insulin sensitivity during both treatment durations, respectively (Zhu *et al*, 2010). Similarly, studies investigating markers of inflammation related to the development of insulin resistance and T2D have shown that fish protein hydrolysates were able to attenuate and produce a sharp decrease in levels of serum high-sensitivity C-reactive protein (hs-CRP), free fatty acids and cytochrome P450 (Asfandiyarova *et al*, 2016).

The translation of biological activities between *in vitro* and *in vivo* situations can present challenges, especially towards oral bioavailability of peptides. The studies highlighted here present a directed approach to tackling obesity and diabetes using hydrolysed marine proteins with multifaceted physiological activity. This study aims to identify if the source of protein including the hydrolysis method affects the bioavailability using a genetic model of obesity and diabetes. Here we deployed a twice daily oral administration of either boarfish or salmon skin gelatine (50 mg/kg bw) hydrolysate that were hydrolysed using the same enzymatic preparation, Alcalase and Flavourzyme in combination (2.4L/500L). Groups of *ob/ob* mice were administered physiological saline alone or in combination with boarfish, salmon skin gelatine (SSG) or metformin and assessed over 30 days. Additionally, the effects of the treatments on glucose homeostasis and markers of obesity and diabetes status were examined.

Background & Aims

Longterm intake of supplemental protein, or dietary modification increasing protein intake from foodstuffs is associated with a reduction in total caloric intake, improvements in body mass index and

anthropometric outcomes. More recently, the effects of longterm protein intakes on satiety and post-prandial glucose excursion have been investigated for the effect upon incretin hormone secretion, protein-protein interactions within the GUT, and secretion of hormones that control GUT-brain satiety centres. The effect of protein hydrolysates on these biological outcomes is poorly understood, with no definitive outcomes in place to make firm nutritional recommendations. For this reason, the aim of this chapter is to assess the effect of chronic twice-daily oral intake of two protein hydrolysate sources identified from previous chapters, and their effect upon food intake and non-fasting blood glucose parameters over a 30 day period in genetically obese, diabetic mice. Finally, terminal analysis investigating longterm markers of obesity, diabetes and bone health will be investigated. This chapter's hypothesis suggests that food intake will be reduced over the duration of the study, improving longterm markers of diabetes control such as HbA_{1c}.

6.2 Materials and Methods

6.2.1 Genetically-induced obese-diabetic (*Ob/Ob*) mice (B6.Cg-Lepob/J)

Mice (12 week old) were ordered from Envigo, Blackthorn, UK. Mice were housed in an air conditioned room maintained within a narrow range of $22 \pm 2^\circ\text{C}$ with a 12 h light: 12 h dark cycle. Drinking water and standard rodent diet was freely available. All animal studies were performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

6.2.2 Glucose tolerance tests (intraperitoneal and oral)

Blood samples were measured from a minor tail vein bleed in fasted (8 h) *ob/ob* mice. Blood glucose was measured using a handheld glucometer (Bayer Contour, Leverkusen, Germany) prior ($t=0$) to administering a glucose challenge (18.8 mmol/kg/ bodyweight). Once administered, tail blood glucose was further analysed at 15, 30, 60, 90 and 120 min.

6.2.3 Insulin sensitivity test

Blood glucose concentrations of animals were measured from a minor tail vein bleed in non-fasted mice using a handheld glucometer (Bayer Contour, Leverkusen, Germany). Blood glucose concentrations were measured prior ($t=0$) and following intraperitoneal administration of bovine insulin as 25 U/kg bodyweight (normal NIH Swiss / HsdOlaT0 / C57Blk) or 50 U/kg bodyweight (*ob/ob* mice) at 30 and 60 minute post injection.

6.2.4 Measurement of indirect calorimetry

Following the various treatments for 30 days mice (at day 40) were housed individually in a Complete Laboratory Animal Monitoring System (CLAMS) metabolic chambers (Columbus Instruments, Columbus, Ohio, USA). For more information on CLAMs refer to section 2.4.5.

6.2.5 Tissue excision

On completion of terminal experimentation mice were fasted (4 h) and sacrificed. Mice were placed unconscious via oral inhalation of a general anaesthetic (Isoflurane) and euthanized via cervical dislocation. The pancreas from each animal was excised removing any extraneous material. The pancreas was sectioned in half from the pancreas head to tail with half being wrapped in aluminium foil and snap frozen in liquid nitrogen and later stored at -70°C and the remaining half being placed into tissue fixative solution (4% paraformaldehyde) for later use in tissue processing and for immunohistochemistry.

6.2.6 Pancreatic hormone content

Thawed tissue was rinsed in cold PBS before being weighed and transferred to a Bijou tube where 2 ml of ice cold acid ethanol (1.5% (v/v) HCl, 75% (v/v) ethanol, 23.5% (v/v) H_2O) was added. The Bijou was then placed on ice for 30 min before being homogenized for 30 sec. The Bijou was blitzed again for 30 second intervals for a total of 2 minutes per pancreas. The homogenate containing Bijou was kept on ice until spun at 4000 rpm for 20 min with the resulting supernatant transferred to a fresh tube. Total volume was then increased by addition of 5 ml of TRIS-HCL (pH 7.4) and placed into a speed vac concentrator (45°C for 2 h) until complete liquid evaporation. The remaining sample extract was reconstituted in 500 μl of TRIS-HCL (pH 7.4) and stored in 1.5 ml Eppendorf tubes at -80°C until analysis.

6.2.7 Assessment of pancreatic insulin and glucagon content

Pancreatic homogenate was diluted to a range of concentrations (1:100, 1:200, 1:500 and 1:1000) using stock RIA buffer prepared in section 2.2.2 and 2.2.3. Pancreatic glucagon content was assessed using a chemiluminescent sandwich ELISA (Millipore, Ontario, Canada) and run according to manufacturer instructions as per section 2.4.8.

6.2.8 Total pancreatic protein content

The Bradford assay was used for protein quantification using diluted samples. Bradford reagent was used as the blank control and is used to subtract the baseline absorbance as described in section 2.4.9.

6.2.9 Assessment of terminal plasma lipid profile

Terminal plasma sample lipid profiles were determined using an I-Lab 650 clinical chemistry system (Instrumentation Laboratory, Warrington, UK). The analysis consisted of assessing total triglycerides, total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) concentration. Reagents for triglyceride analysis were obtained from Instrumentation Laboratory (Warrington, UK) and reagent for LDL cholesterol were obtained from Randox Healthcare (Randox, Co. Antrim, NI).

6.2.10 Statistical analysis

Data was expressed as mean \pm SEM with values compared using one-way analysis of variance (ANOVA) followed by *post-hoc* students T-test unless otherwise stated. Data between groups were considered statistically significant if $p \leq 0.05$. Data was plotted and analysed using Graphpad Prism 5.

Results

Fig 6.1 – Grouping of mice prior to long-term hydrolysate investigation

Genetically obese and diabetes mice (*ob/ob*) were initially grouped depending on non-fasting blood glucose taken in the morning and on the basis of bodyweight. There was no significant difference in either parameter between each group of mice (Fig. 6.1).

Fig 6.2 – Effect of twice daily treatment on non-fasting blood glucose parameters

Mice were administered their assigned treatments twice daily, once in the morning (9.00 am) and once in the afternoon (5.00 pm). Every third day non-fasting blood glucose (NFBG) was measured via a handheld glucometer (Bayer, UK). Treatments consisted of orally administered physiological saline only (as control) or saline containing Boarfish protein hydrolysate (BPH, 50 mg/kg/bw), or Salmon skin gelatine (SGPH, 50 mg/kg/bw) or Metformin (200 mg/kg/bw). Metformin initially resulted in a significant reduction of NFBG at day 3 ($p < 0.05$) however the effect was lost until day 12 ($p < 0.05$) and was sustained throughout the study up until day 27 ($p < 0.05$ – $p < 0.001$). BFPH significantly lowered NFBG at day 6 ($p < 0.05$) and this was sustained throughout up until completion at day 27 ($p < 0.05$ – $p < 0.001$). The salmon skin gelatin (SSG) protein hydrolysate showed sustained lowered NFBG from day 9 ($p < 0.05$) and throughout the study until completion at day 27 ($p < 0.05$ – $p < 0.001$), compared to the *ob/ob* saline only treated control group. All groups resulted in a significant total reduction of total NFBG when analysed via AUC, with each *ob/ob* treatment group on average causing a 40% reduction ($p < 0.01$ – $p < 0.001$) compared to the saline treated *ob/ob* control group. (Fig 6.2).

Fig 6.3 – Effect of twice daily treatment on non-fasting plasma insulin parameters

Every third day non-fasting plasma insulin (NFGB) was measured via RIA. Generally, there was an overall increasing trend in plasma insulin concentrations, however only Boarfish at day 12 has shown a significant increase ($p < 0.05$) versus the *ob/ob* saline control. When analysed via total insulin area under the curve (AUC), again only the BPH group resulted in a significant increase in NF-plasma insulin concentrations versus the *ob/ob* saline control group, however it is noted, while non-significant for the other treatment groups such as SSG and metformin, there was an increased plasma insulin trend. (Fig 6.3).

Fig 6.4 – Effect of twice daily treatments on weight parameters

Mice were weighed every third day. On completion of the study, no intergroup significance was reported in weight gain. On average, each *ob/ob* treatment group had weight increases of approximately 11 – 14%, which was not significantly differently to the *ob/ob* saline control (Fig 6.4).

Fig 6.5 A – Effect of twice daily treatments on dietary energy intake

Food was measured every 3 days of which animals had ad-lib access to. The weight of the food was measured and cumulatively scored with each gram of food eaten consisting of 12.99 Kj. The metformin group showed a significant reduction of energy intake from day 9 through completion of the study at day 27 ($p < 0.05$ – $p < 0.001$). The Boarfish and SSG groups showed a significant energy intake reduction from day 12 throughout the remaining of the study at day 27 ($p < 0.05$ – $p < 0.001$), with except for the SGPH group at day 15. (Fig 6.5 A).

Fig 6.5 B – Effects of twice daily treatment on post-treatment fasting blood glucose levels

Following twice daily treatments, animals were fasted for 8 h and blood glucose concentrations determined using a handheld glucometer from a tail vein bleed. No significant difference in fasting blood glucose concentration was noted in SGPH or Metformin treated animals, however a significant improvement was noted within the BPH treated animals with an 8 mmol/L reduction (40% ?? $p < 0.05$) versus the *ob/ob* saline treated control group. (Fig 6.5 B).

Fig 6.6 – Effects of twice daily treatment on terminal oral glucose tolerance in mice

Following 27 days of treatment glucose tolerance was investigated after fasting animals for 8 h. Fasting blood glucose levels were established prior to oral glucose administration ($t=0$ min) and at regular intervals up to 120 min. Significant blood glucose improvements were noted at 15 min post glucose challenge in the metformin group ($p < 0.05$), BPH ($p < 0.05$) and the SGPH ($p < 0.001$) compared to the *ob/ob* saline control. The effect was lost in each group from 30 min and 60 min, however at 90 min a significant reduction in blood glucose levels were reported in the metformin ($p < 0.05$) and SGPH ($p < 0.01$) compared to the *ob/ob* saline control. Interestingly, all treatment groups at 120 min had improved glucose parameters ($p < 0.05$) versus *ob/ob* saline treated control, respectively. When analysed via total blood glucose AUC, only SGPH ($p < 0.001$) and Metformin ($p < 0.05$) groups presented a significant total reduction versus *ob/ob* saline control. (Fig 6.6).

Fig 6.7 – Effects of twice daily treatment on terminal ip glucose tolerance

Following treatment analysis on ip glucose tolerance (ip GGT) was investigated after fasting mice for 8 h. Fasting blood glucose levels were established prior to oral glucose administration ($t=0$ min) and at regular intervals up to 120 min. There was no improvement in glucose tolerance noted in the *ob/ob* treatment groups versus *ob/ob* saline control between 15 min

and 90 min. However, the BPH group resulted in a significant reduction in blood glucose concentration at both 90 min ($p<0.05$) and 120 min ($p<0.001$), furthermore, metformin also had shown reduction in blood glucose levels at 120 min ($p<0.001$) versus the *ob/ob* saline control group. Using analysis of total blood glucose from AUC data, only boarfish ($p<0.01$) and metformin ($p<0.01$) showed a significant improvement in total glucose reduction over the 120 min versus the *ob/ob* saline control, respectively. (Fig 6.7)

Fig 6.8 – Effect of twice daily treatment on terminal insulin sensitivity

Each group was fasted for 4 h prior to insulin injection (50 U/kg/bw bovine insulin). Blood glucose concentration was established prior to insulin injection ($t=0$ min) and at 30 min and 60 min post-injection. There was no significant improvements in insulin sensitivity noted between any of the *ob/ob* treatment groups compared to the *ob/ob* saline control, however a trend towards an improvement was noted in the BPH group, but this failed to reach significance. (Fig 6.8).

Fig 6.9 B – Effects of twice daily treatments on long-term markers of glucose regulation via glycated haemoglobin analysis (HbA1c)

Using a HbA1c assay (A1cNow), glucose control was established using this long-term marker. Each of the treatment groups had shown a significant reduction in HbA1c versus the *ob/ob* saline treated control group. A reduction within the BPH of 2.7 % ($p<0.001$), SGPH of 2.2 % ($p<0.01$) and metformin group of 2.1% ($p<0.01$). (Fig 6.9 B).

Fig 6.10 – Effects of twice daily treatment on non-fasting circulating total GLP-1 concentration

Terminal plasma circulating GLP-1 was established using the chemiluminescent MSD mesoplex assay. There was no change found between the SGPH treatment group and the *ob/ob* saline control group. However, there was a significant increase in circulating total GLP-1 concentration in the BPH ($p < 0.001$) whereas the opposite effect was noted within the Metformin group, which displayed a significant reduction ($p < 0.001$) versus the *ob/ob* saline control. (Fig 6.10).

Fig 6.11 A – Effects of twice daily chronic treatment on circulating plasma cholesterol

Plasma lipids were analysed using a clinical lab 650 analyser. Cholesterol concentrations were significantly lower in both the SSGPH ($p < 0.05$) and the metformin ($p < 0.01$) treatment groups versus the *ob/ob* saline control. The Boarfish treatment group failed to reach significance ($P > 0.05$). (Fig 6.11 A).

Fig 6.11 B – Effects of twice daily chronic treatment on circulating plasma HDL cholesterol

Plasma lipids were analysed using a clinical lab 650 analyser. High-density lipoprotein cholesterol concentrations within the three *ob/ob* treatment groups were unchanged versus the *ob/ob* saline control. As expected the lean saline treated control group, had a marked reduction of circulating HDL versus that of the *ob/ob* saline treated control group ($p < 0.001$). (Fig 6.11 B).

Fig 6.12 A – Effects of twice daily chronic treatment on circulating plasma triglycerides

Triglyceride concentrations were normalised towards the lean control values and were significantly lower in the BPH ($p < 0.05$), SSGPH ($p < 0.01$) and the metformin ($p < 0.001$) treatment groups when compared to the *ob/ob* saline treated control group. (Fig 6.12 A)

Fig 6.12 B – Effects of twice daily treatment on circulating plasma low-density lipoprotein levels analysed post-study

LDL concentrations were normalised towards the lean control values and were significantly lower in the BPH SSGPH and the metformin (all $p < 0.001$) treatment groups when compared to the *ob/ob* saline control group. (Fig 6.12 B).

Fig 6.13 A/B – Effects of twice daily chronic treatment on acute insulin secretion from isolated pancreatic islets and islet insulin content

Pancreatic functionality was assessed after isolating pancreatic islets from each treatment group and testing insulin secretion and insulin content following acute treatment with known insulin secretagogues. Isolated islets responded to known secretagogues in a glucose dependent manner (Fig. 6.13A). Analysis showed a similar functionality between each treatment group, as well as similar insulin content as assessed via RIA analysis. No significant differences were reported between the various *ob/ob* treatment groups. (Fig 6.13 A/B)

Fig 6.14 A – Changes in pancreatic protein and hormone contents following the chronic study

Pancreatic hormone changes were established using acid-ethanol extraction and homogenisation of pancreatic tissue and subsequently analysed using a Bradford protein assay. A significant reduction of protein content was evident within the BPH ($p < 0.01$) and SSGPH ($p < 0.05$) versus the *ob/ob* saline control group. No changes in total protein content were noted within the metformin group, respectively. (Fig 6.14 A)

Fig 6.14 B – Total pancreatic insulin content expressed per mg of wet tissue

Following acid ethanol extraction, insulin content was analysed using RIA, no changes in pancreatic insulin content was noted between *ob/ob* treatment groups compared to the *ob/ob* saline control group. (Fig 6.14 B).

Fig 6.19 B – Immunohistochemistry analysis of average islet cell area of excised pancreas in *ob/ob* treatment groups

Islets were stained for glucagon, insulin and cellular nuclei stain DAPI. Based on total cell morphology, islets were assessed for size via cell area analysis. A significant reduction in cell area was reported within the BPH ($p < 0.001$) and the metformin ($p < 0.001$) treatment groups versus the *ob/ob* saline control group. (Fig 6.19 B).

Fig 6.20 A – Immunohistochemistry analysis of average insulin β -cell area of excised pancreas in *ob/ob* treatment groups

Islets were stained for insulin. Based on total insulin cell area morphology, islets were assessed for total insulin cell area. A significant reduction in insulin cells were reported in the BPH ($p < 0.01$) and metformin ($p < 0.001$) whereas the SSGPH displayed a significant increase in insulin cell area ($p < 0.05$) versus the *ob/ob* saline treatment group. (Fig 6.20 A).

Fig 6.20 B – Immunohistochemistry analysis of average glucagon α -cell area of excised pancreas in *ob/ob* treatment groups

Pancreatic islets were stained for glucagon. Based on total glucagon cell area morphology, islets were assessed for total glucagon cell area analysis. A significant reduction in glucagon cells were reported in the BPH ($p < 0.001$) and SGPH ($p < 0.001$) versus the *ob/ob* saline treatment group. No change in glucagon cell area was reported within the metformin group versus the *ob/ob* saline control group. (Fig 6.20 B).

Fig 6.21 A - Immunohistochemistry analysis of total insulin cell area of excised pancreas in *ob/ob* treatment groups

Pancreatic islets were stained for insulin and quantified based on total pancreatic insulin cell area. A significant increase in total insulin cells were shown in the BPH ($p < 0.001$) whereas a reduction was noted within the SSGPH ($p < 0.001$) and metformin ($p < 0.01$) treatment groups versus the *ob/ob* saline treatment group. (Fig 6.21 A).

Fig 6.21 B - Immunohistochemistry analysis of total glucagon cell area of excised pancreas in *ob/ob* treatment groups

Pancreatic islets were stained for glucagon and quantified based on total pancreatic glucagon cell area. A significant reduction in total glucagon cell area were reported in the BPH ($p < 0.001$), SGPH ($p < 0.001$) and metformin ($p < 0.001$) versus the *ob/ob* saline treated group. (Fig 6.21 B).

Fig 6.22 - Immunohistochemistry analysis of total alpha-cell to beta-cell ratio in *ob/ob* treatment groups

Total glucagon to insulin cell ratio were analysed to complete the pancreatic cell morphology changes post-treatment. A significant reduction in α - to β - cell ratio was noted within the BPH ($p < 0.001$) and SSGPH ($p < 0.001$) whereas interestingly a significant increase in this ratio was noted within the metformin ($p < 0.001$) treatment group compared to the *ob/ob* saline treated control group. (Fig 6.22)

Fig 6.23 A – Bone mineral density parameter changes post-treatment analysed using DEXA

No changes in BMD were reported in any of the *ob/ob* treatment groups versus the *ob/ob* saline control group. (Fig 6.23 A)

Fig 6.23 B – Bone mineral content parameter changes post-treatment analysed using DEXA

Using DEXA analysis, a significant increase in BMC was noted in both the BPH ($p < 0.01$) and SSGPH ($p < 0.01$), whereas a reduction was noted in the metformin ($p < 0.05$) group versus the *ob/ob* saline control group, respectively. Fig 6.23 B).

Fig 6.24 A – Body composition changes in total lean mass post-treatment analysed via DEXA

No changes in total body lean mass were noted between each of the *ob/ob* treatment groups compared to the *ob/ob* saline treated control group. (Fig 6.24 A).

Fig 6.24 B – Body composition changes in total fat mass post-treatment analysed via DEXA

No changes in total body fat mass were noted between each of the *ob/ob* treatment groups compared to the *ob/ob* saline treated control group. As expected in the lean saline treated control group, total fat mass was significantly lower than each of the *ob/ob* treatment groups ($p < 0.001$) (Fig 6.24 B).

Fig 6.25 A - Body composition changes in total fat % post-treatment analysed via DEXA

No changes in total % body fat were noted between each of the *ob/ob* treatment groups compared to the *ob/ob* saline treated control group. (Fig 6.25 A).

6.25 B - Body composition changes in total lean mass versus fat mass post-treatment analysed via DEXA

No changes in total lean mass were noted between each of the *ob/ob* treatment groups compared to the *ob/ob* saline control group. (Fig 6.25 B).

6.26-30 – Changes in oxygen exchange (VO_2 , VCO_2) and respiratory exchange ratio (RER) analysed post-treatment using CLAMS

No changes in oxygen exchange of O_2 or VO_2 including RER were noted post-treatment when compared to the *ob/ob* saline treated control group. (Fig 6.26 – 6.30).

Fig 6.1

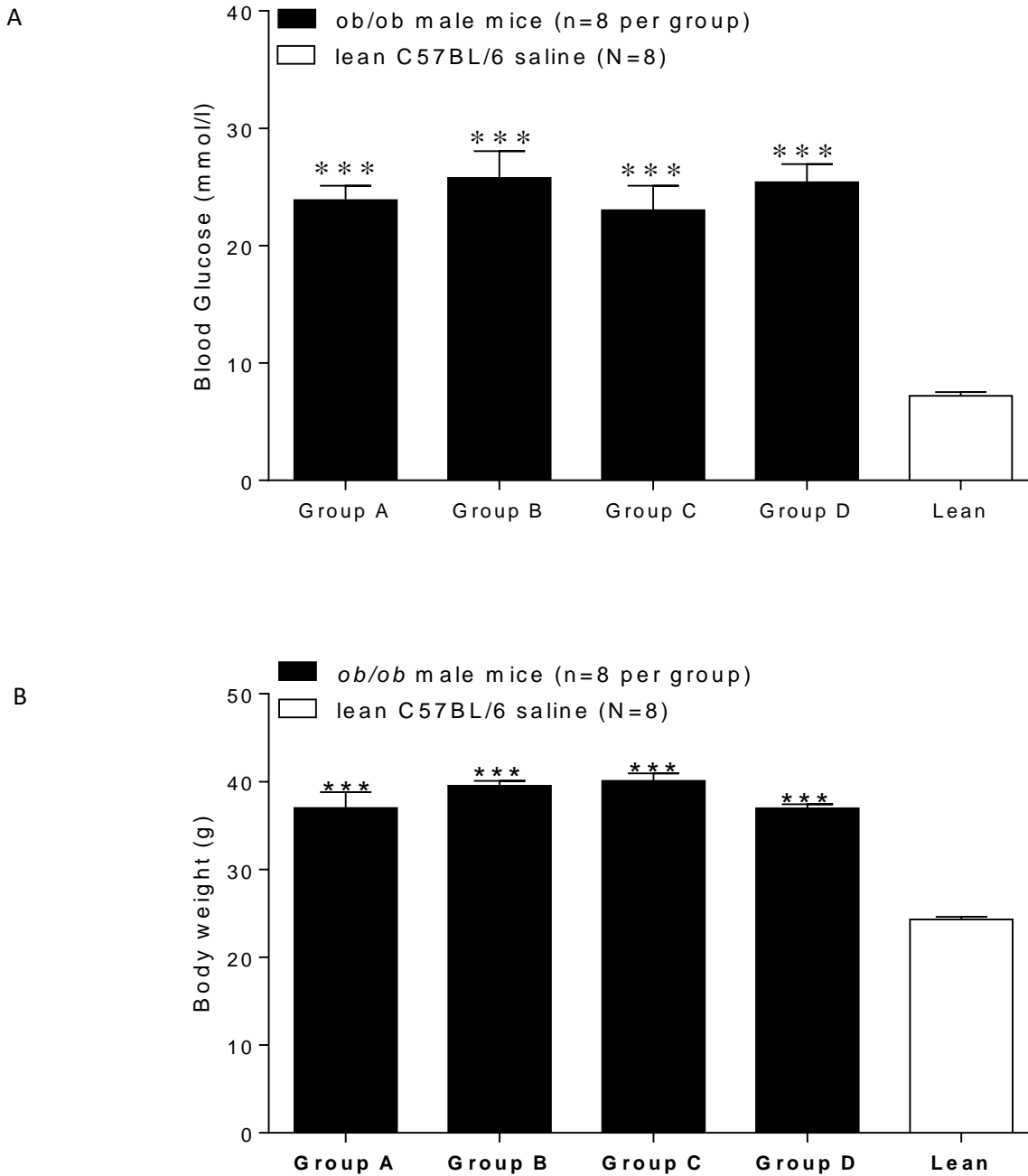


Fig 6. 1 – Animals were given an acclimatization period before being grouped. Groups consisted of evenly matched non-fasting blood glucose (**A**) and body weight (**B**). Both blood glucose and weight were significantly elevated versus lean control group. Results are Mean \pm SEM for 8 observations per group. *** $p < 0.001$ versus lean control group. No differences displayed between the various *ob/ob* groups of mice prior to the start of the experimentation period.

Fig 6.2

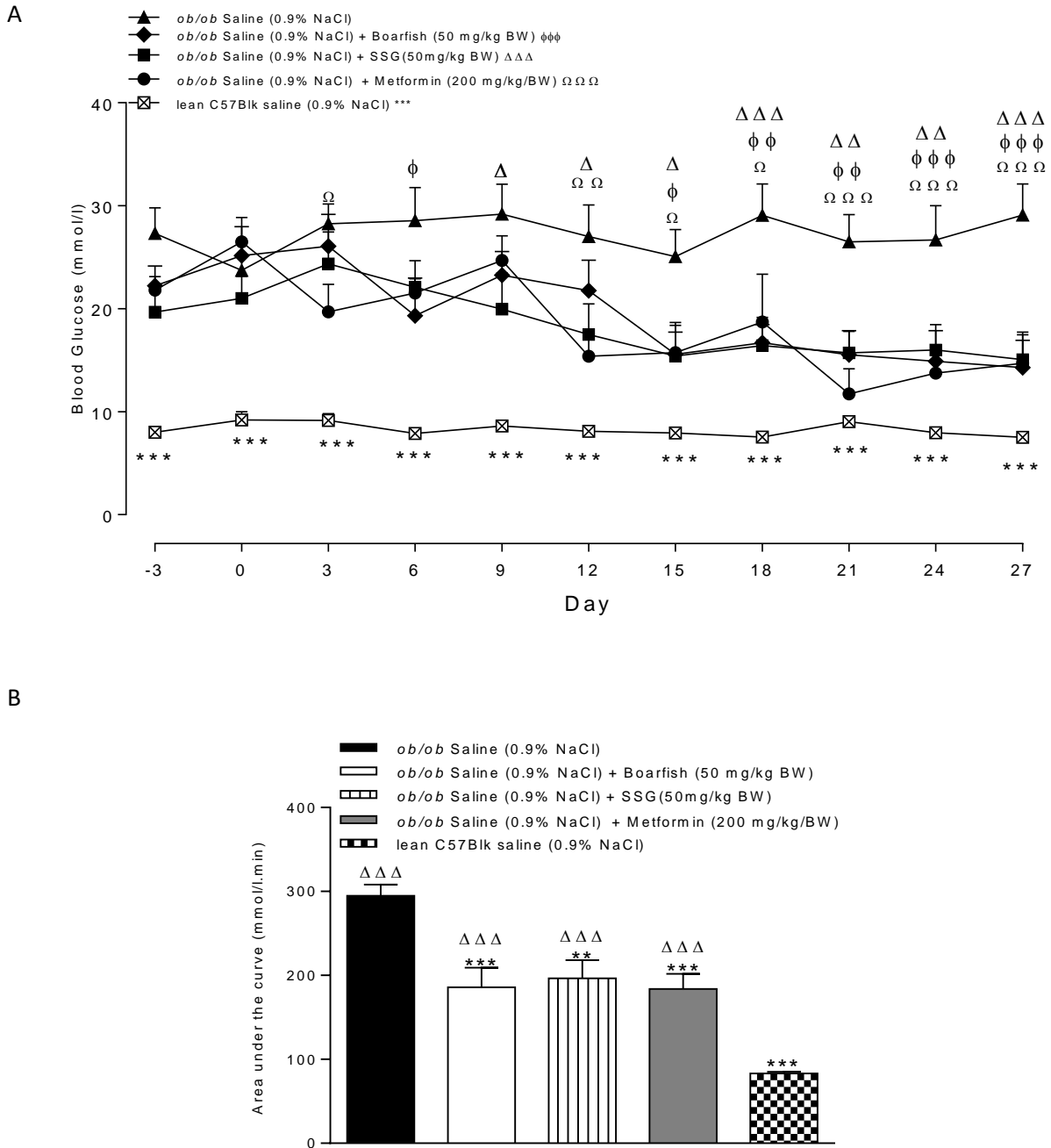
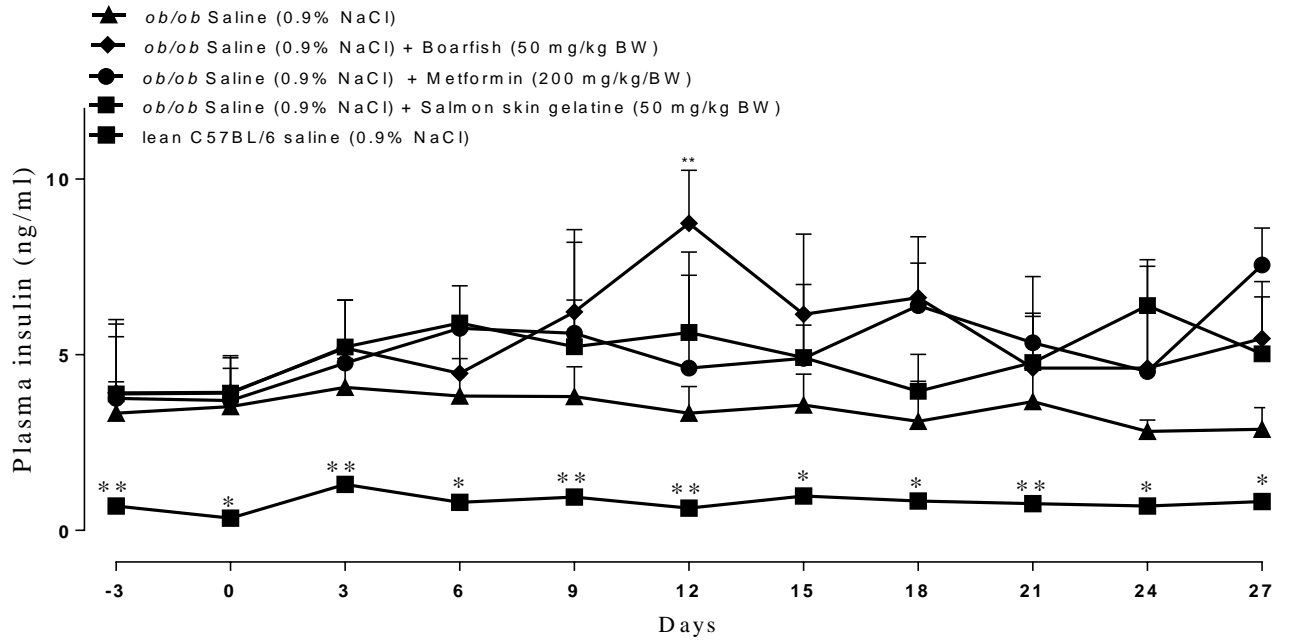


Fig 6.2. Effects of twice-daily administration of marine hydrolysates on blood glucose in *ob/ob* mice **(A)**. Parameters were measured 3 days prior to and 27 days twice daily treatment with marine hydrolysates (50 mg/kg bw), metformin (200 mg/kg bw) or saline vehicle (0.9% (w/v) NaCl). Values represent mean \pm SEM for 8 mice. *** $p < 0.001$ vs *ob/ob* saline, $\Delta p < 0.05$, $\Delta\Delta p < 0.01$, $\Delta\Delta\Delta p < 0.001$ compared to salmon skin gelatine; $\phi p < 0.05$, $\phi\phi p < 0.01$, $\phi\phi\phi p < 0.001$ compared to boarfish; $\Omega p < 0.05$, $\Omega\Omega p < 0.01$, $\Omega\Omega\Omega p < 0.001$ compared to metformin. **(B)** represents Mean \pm SEM blood glucose area under the curve ($AUC_{day\ 0-27}$). *** $p < 0.001$ versus *ob/ob* saline control and $\Delta\Delta\Delta p < 0.001$ versus lean c57blk, respectively.

Fig 6.3

A



B

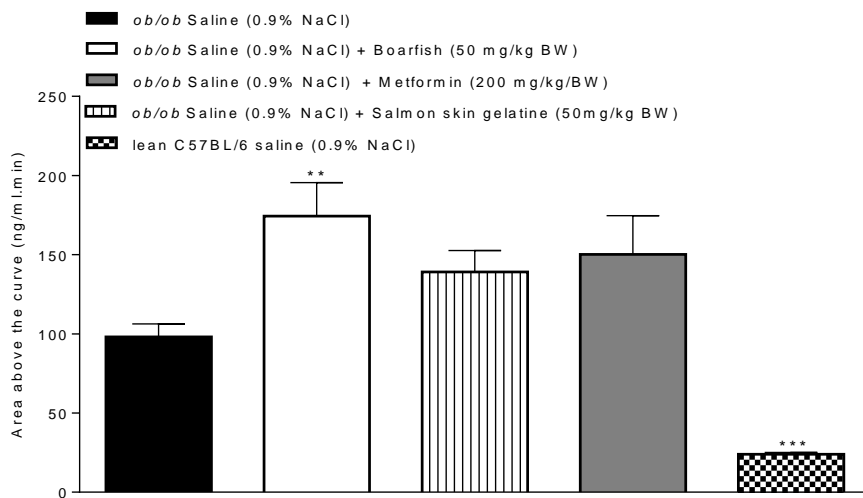


Fig 6.3. Effects of twice-daily administration of marine hydrolysates on circulating plasma insulin in *ob/ob* mice **(A)**. Parameters were measured 3 days prior to and 27 days during twice daily treatment with marine hydrolysates (50 mg/kg bw), metformin (200 mg/kg bw) or saline vehicle (0.9% (w/v) NaCl). Values represent mean \pm SEM for 8 mice. * $p < 0.05$, ** $p < 0.001$ vs *ob/ob* saline, **(B)** represents Mean \pm SEM plasma area above the curve (AAC). ** $p < 0.01$, *** $p < 0.001$, versus *ob/ob* saline control and $\Delta \Delta \Delta p < 0.001$ versus lean c57blk, respectively.

Fig 6.4

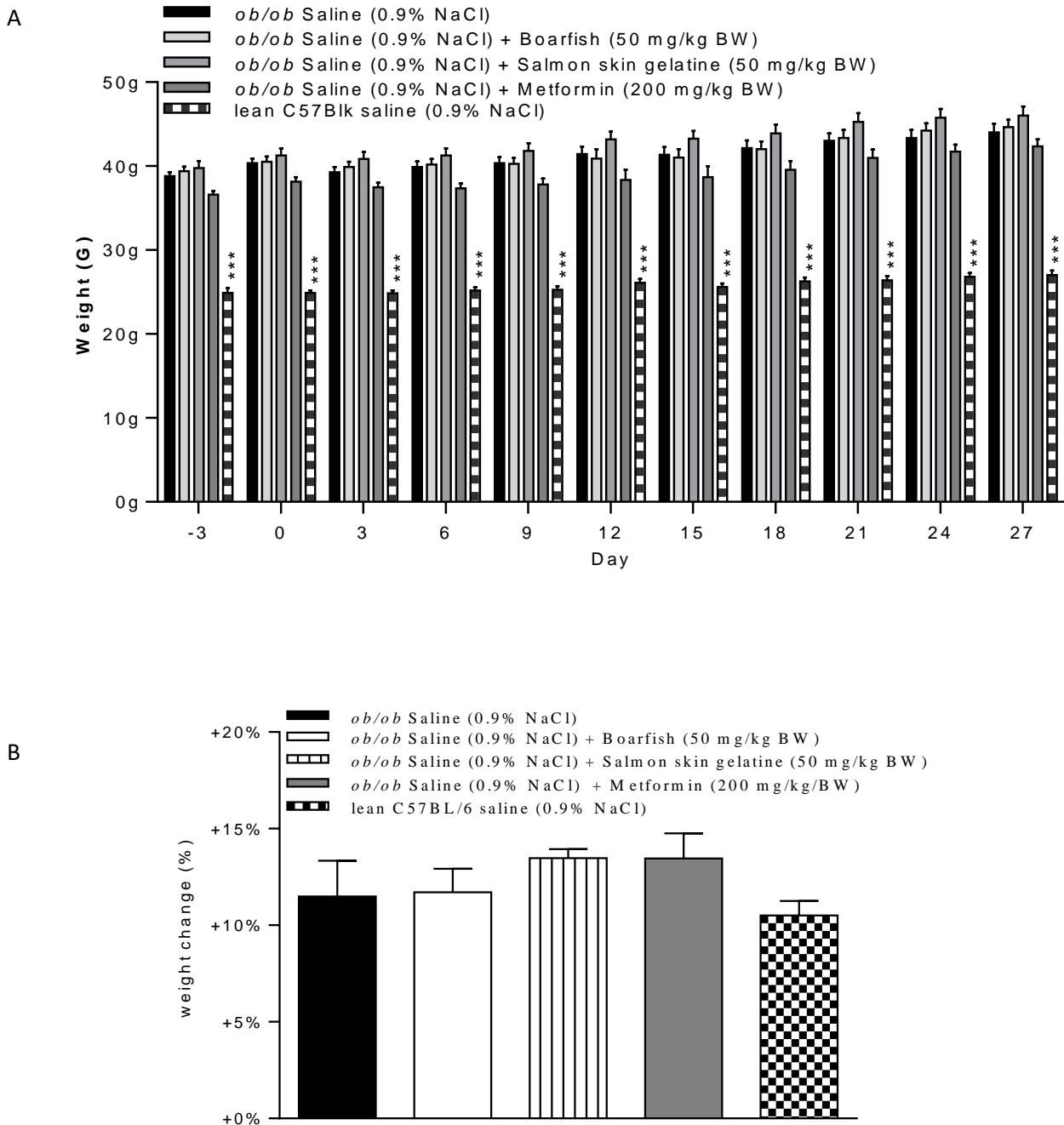


Fig 6.4. Effects of twice-daily administration of marine hydrolysates on weight gain **(A)** and percentage weight change **(B)** in *ob/ob* mice. Weight measured 3 days prior to every 3 days during a twice daily treatment (for 2 days) with marine hydrolysates (50 mg/kg bw), metformin (200 mg/kg bw) or saline vehicle (0.9% (w/v) NaCl). Values represent mean \pm SEM for 8 mice. *** $p < 0.001$ versus *ob/ob* saline control group. **(B)** represents Mean \pm SEM weight change over 27 days of treatment, respectively.

Fig 6.5

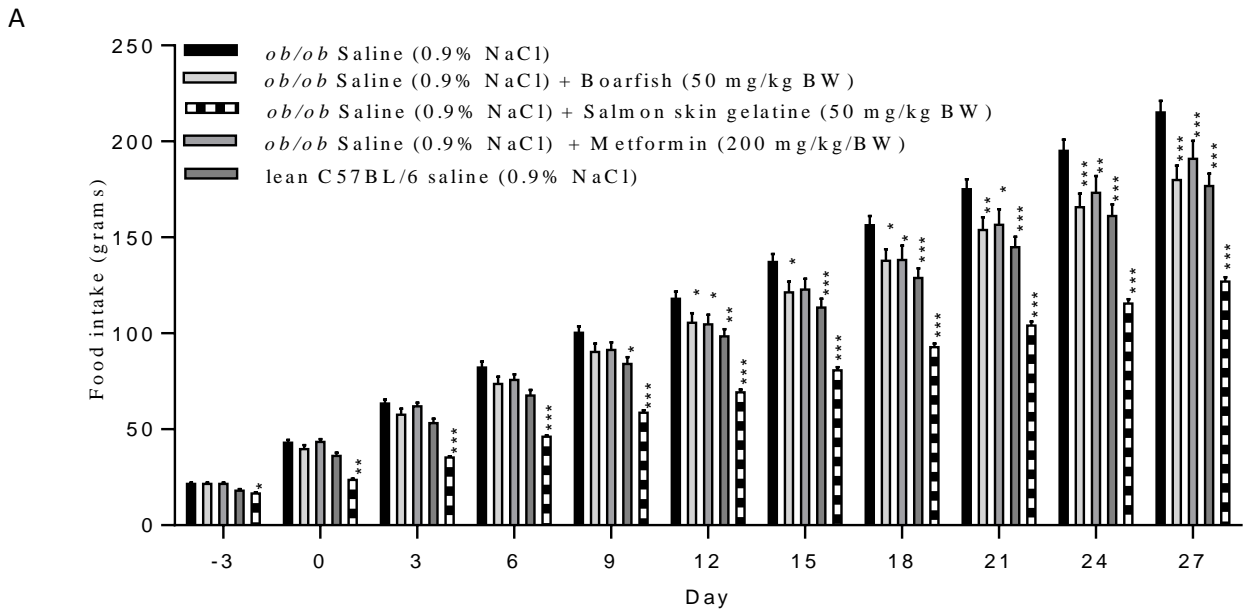


Fig 6.5. (A) Effects of twice-daily administration of marine hydrolysates on food intake in ob/ob mice. Food was measured 3 days prior to and 27 days during twice daily treatment with marine hydrolysates (50 mg/kg bw), metformin (200 mg/kg bw) or saline vehicle (0.9% (w/v) NaCl). Values represent mean \pm SEM for 8 mice. $8p < 0.05$, $**p < 0.01$ and $***p < 0.001$ versus ob/ob saline control group.

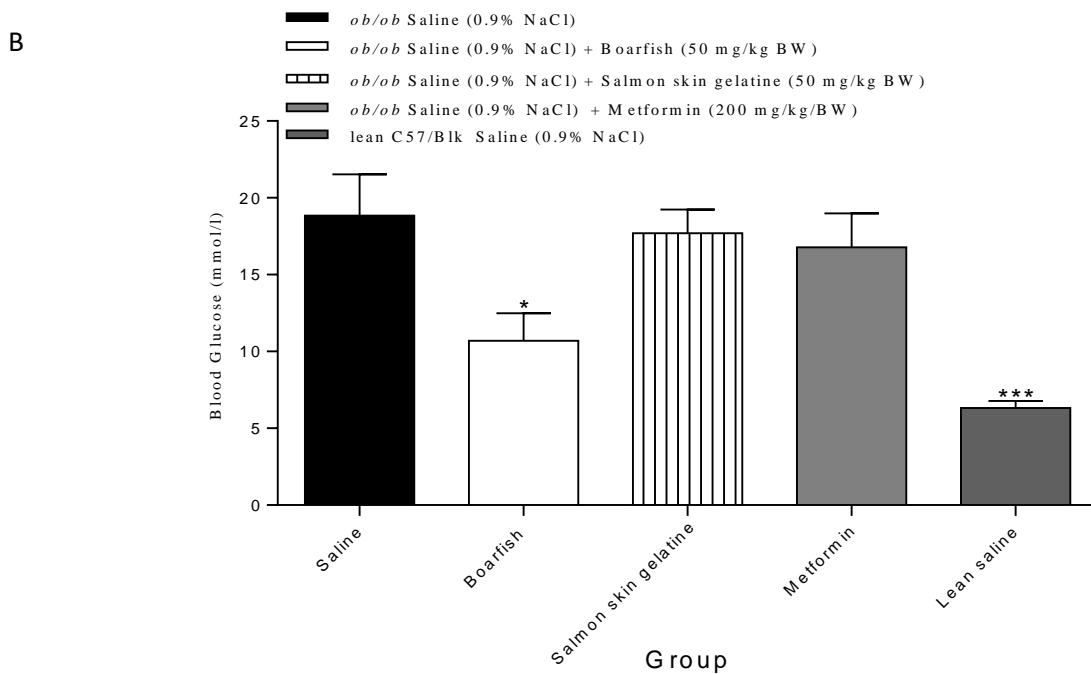


Fig 6.5 (B). Effects of twice-daily administration of marine hydrolysates on fasting blood glucose. Mice were fasted for 12 hours prior to measurement of glucose. Values represent mean \pm SEM for 8 mice. $*p < 0.05$ versus ob/ob saline control group.

Fig 6.6

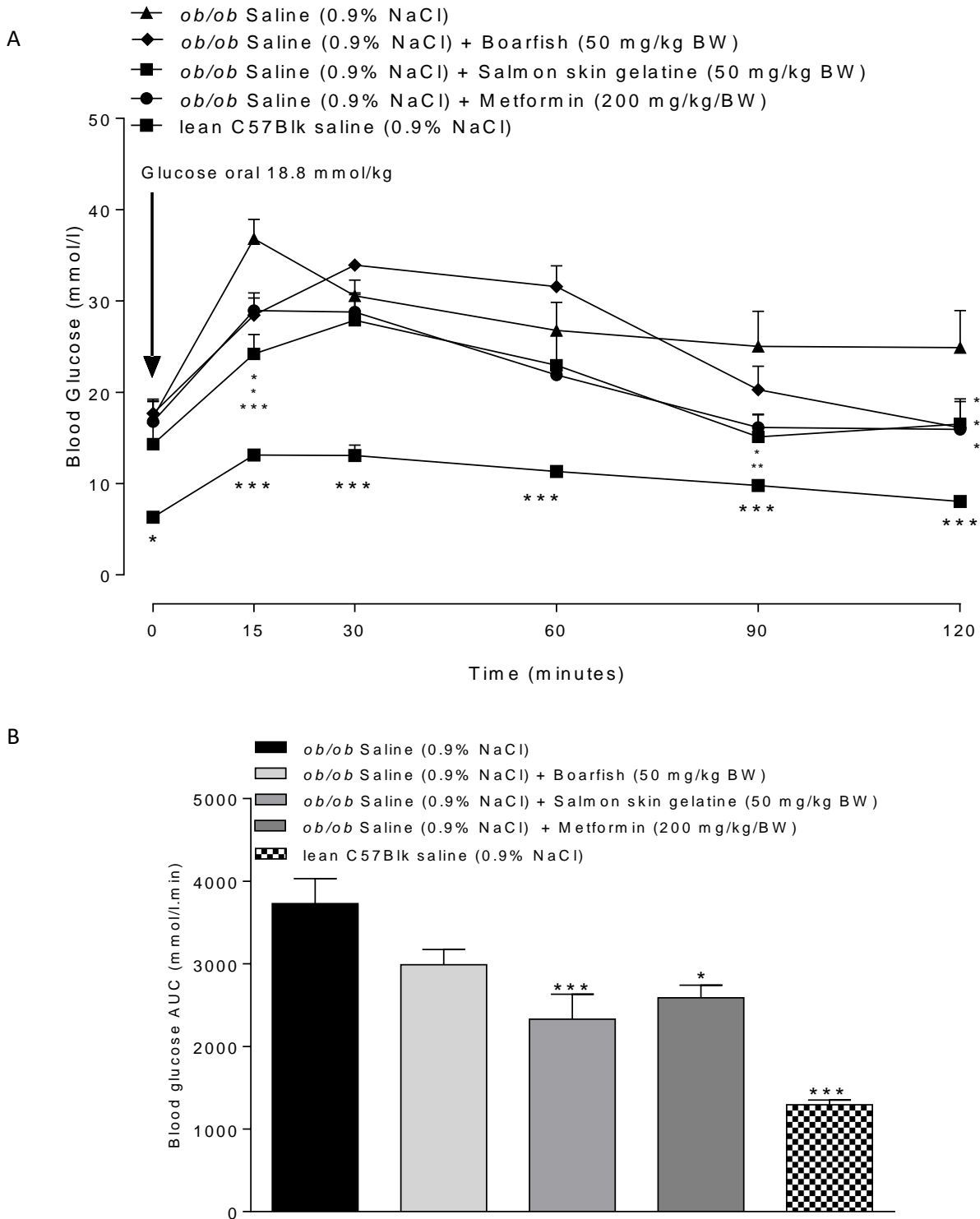


Fig 6.6. Effects of twice-daily administration of marine hydrolysates on **(A)** terminal oral glucose tolerance and **(B)** integrated AUC values. Parameters were measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; oral gavage). **B** - Glucose AUC values for 0-120 min post-injection. Values are expressed as mean \pm S.E.M. for 8 mice. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to *ob/ob* saline treated controls.

Fig 6.7

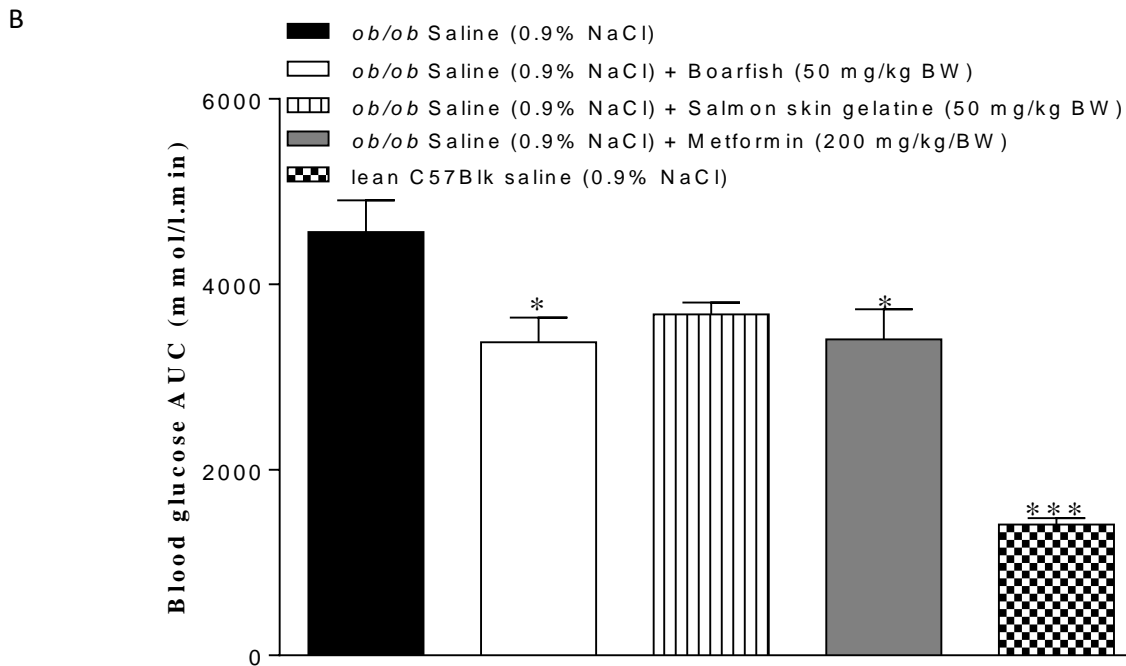
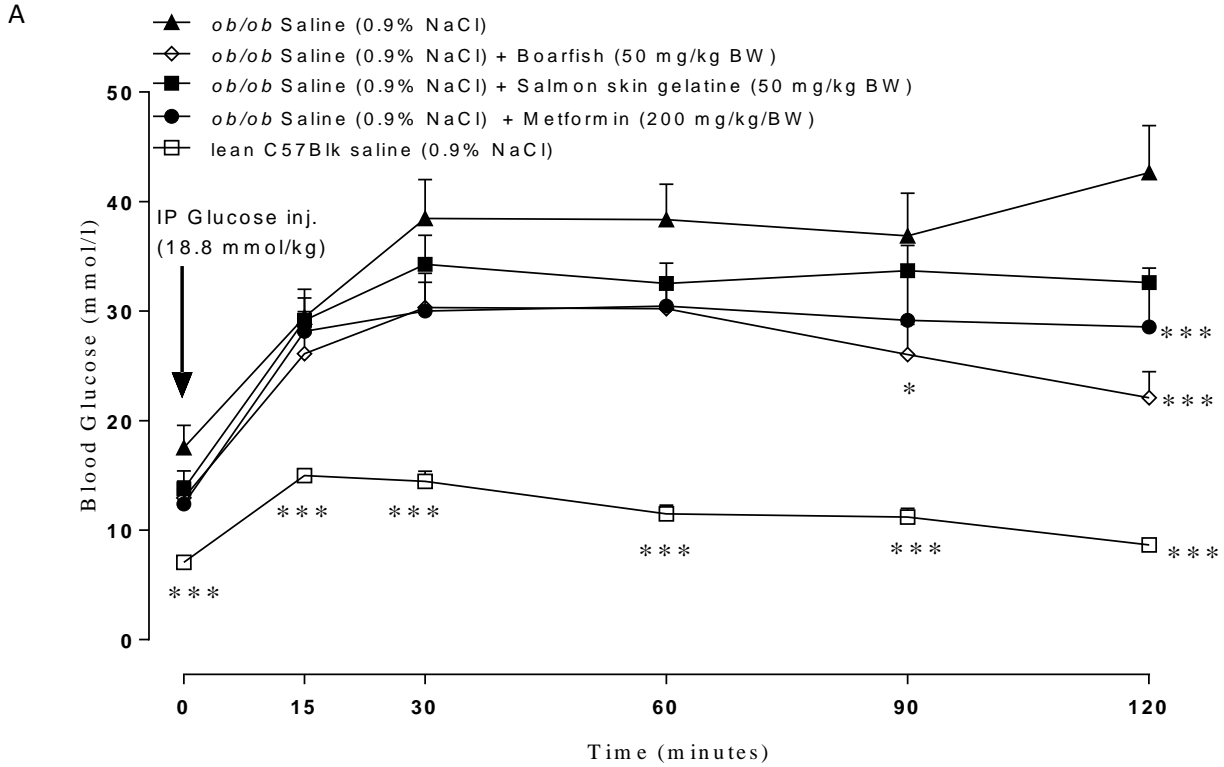


Fig 6.7. Effects of twice-daily administration of marine hydrolysates on **(A)** terminal IP glucose tolerance and **(B)** integrated AUC values. Parameters were measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; IP Injection). **Panel B** - Glucose AUC values for 0-120 min post-injection. Values are expressed as mean \pm S.E.M. for 8 mice. * $p < 0.05$, and *** $p < 0.001$ compared to *ob/ob* saline controls.

Fig 6.8

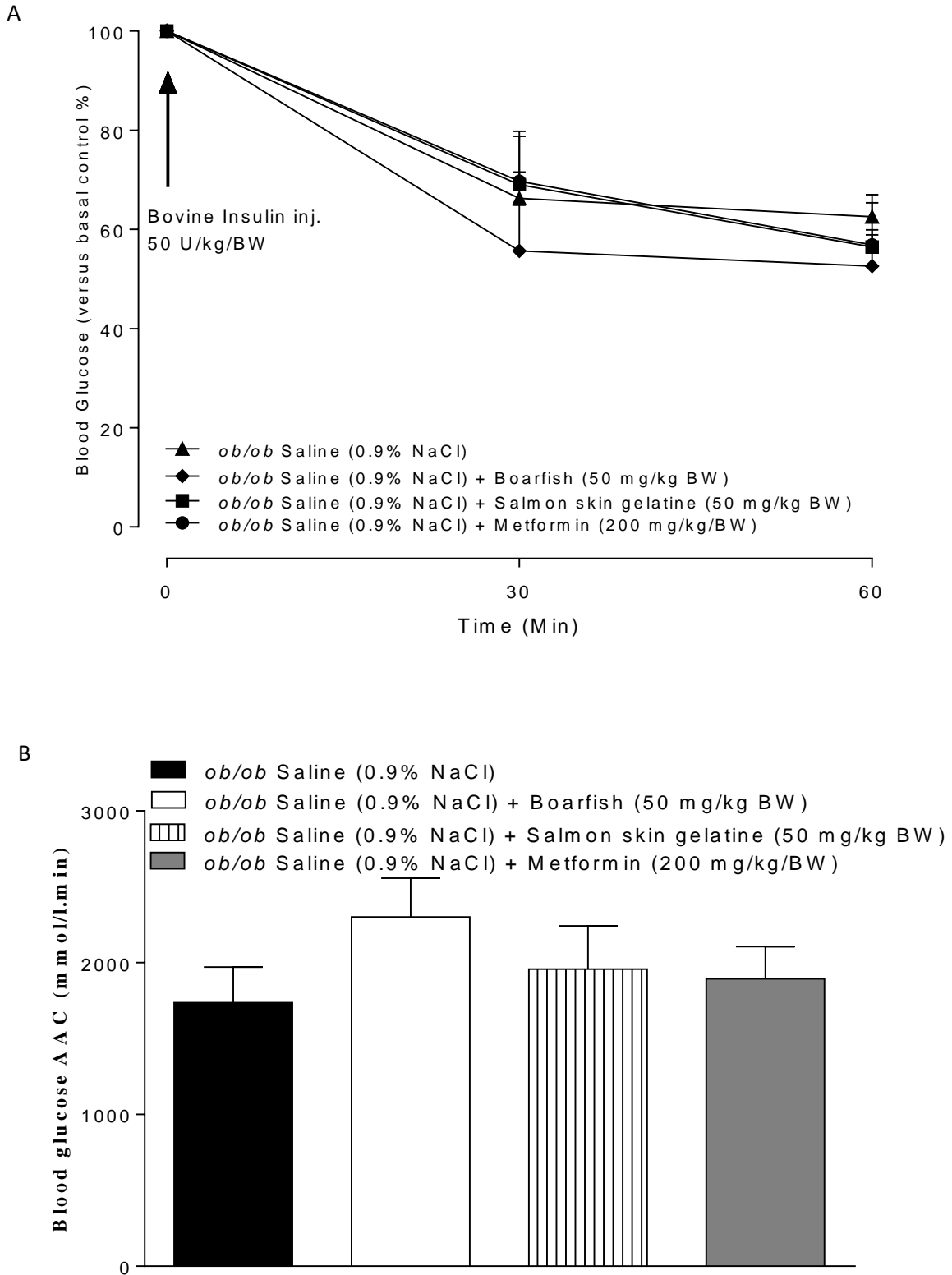


Fig 6.8. Effects of twice-daily administration of marine hydrolysates on **(A)** Insulin sensitivity and **(B)** Mean AAC values. Parameters were measured prior to (t=0) and 30 and 60 min after administration of insulin alone (50 U/kg body weight; IP Injection, Bovine Insulin). Insulin AAC values for 0-60 min post-injection. Values are expressed as mean \pm S.E.M. for 8 mice.

Fig 6.9

A

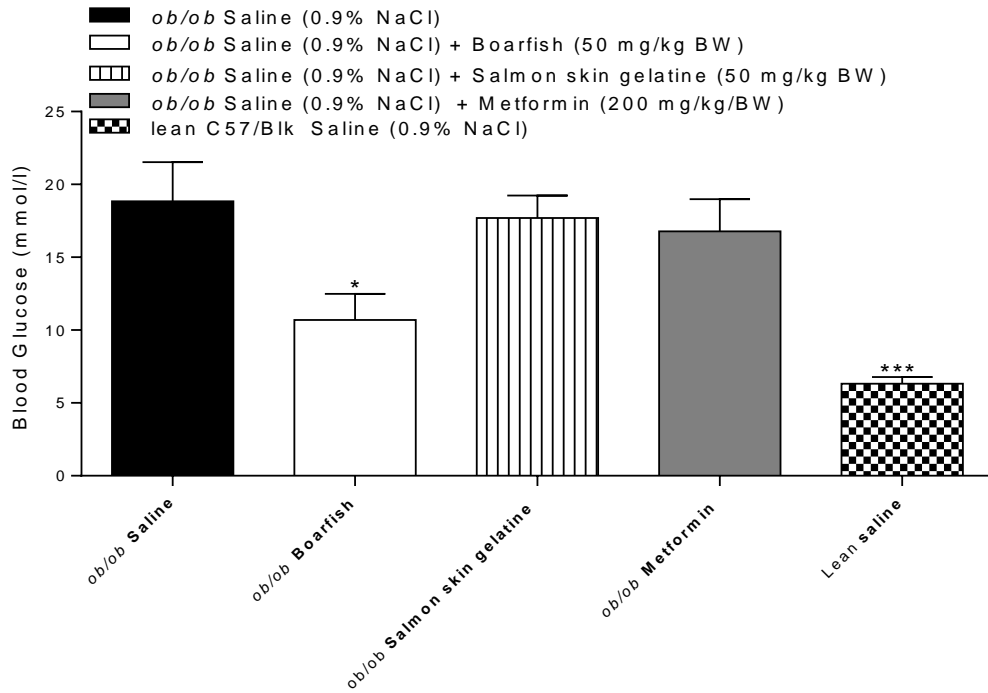


Fig 6.9 (A). Effects of chronic administration of marine hydrolysates on fasting blood glucose. Mice were fasted for 12 hours prior to measurement of glucose. Values represent mean \pm SEM for 8 mice. * $p < 0.05$ versus *ob/ob* saline treated control group.

B

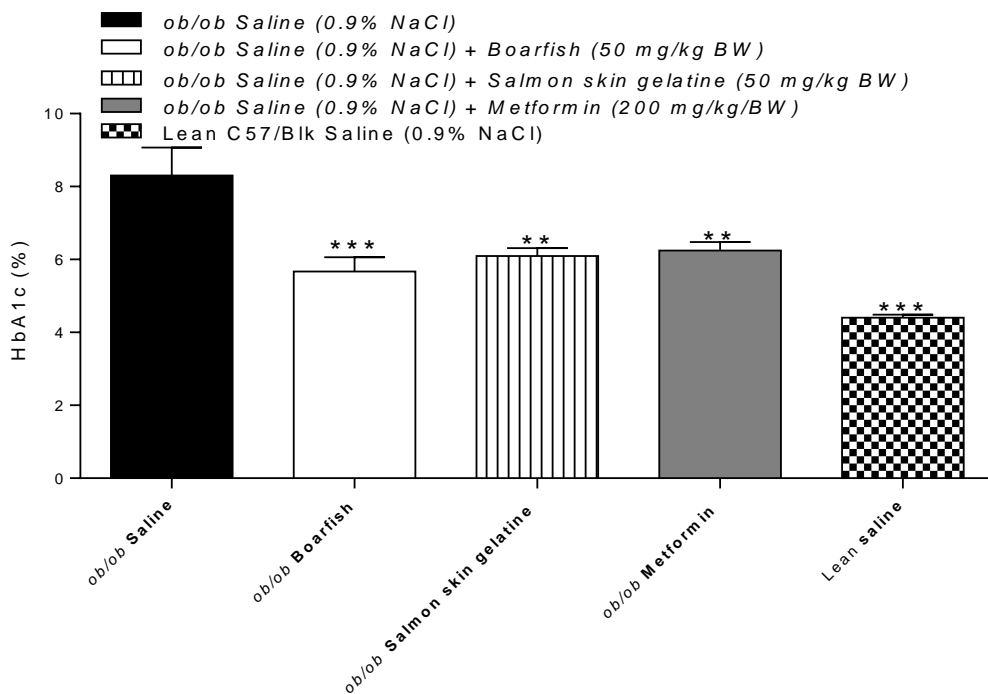


Fig 6.9 B. Effects of chronic administration of marine hydrolysates on terminal glycated haemoglobin (HbA_{1c}). Values represent mean \pm SEM for 8 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus *ob/ob* saline control group.

Fig 6.10

A

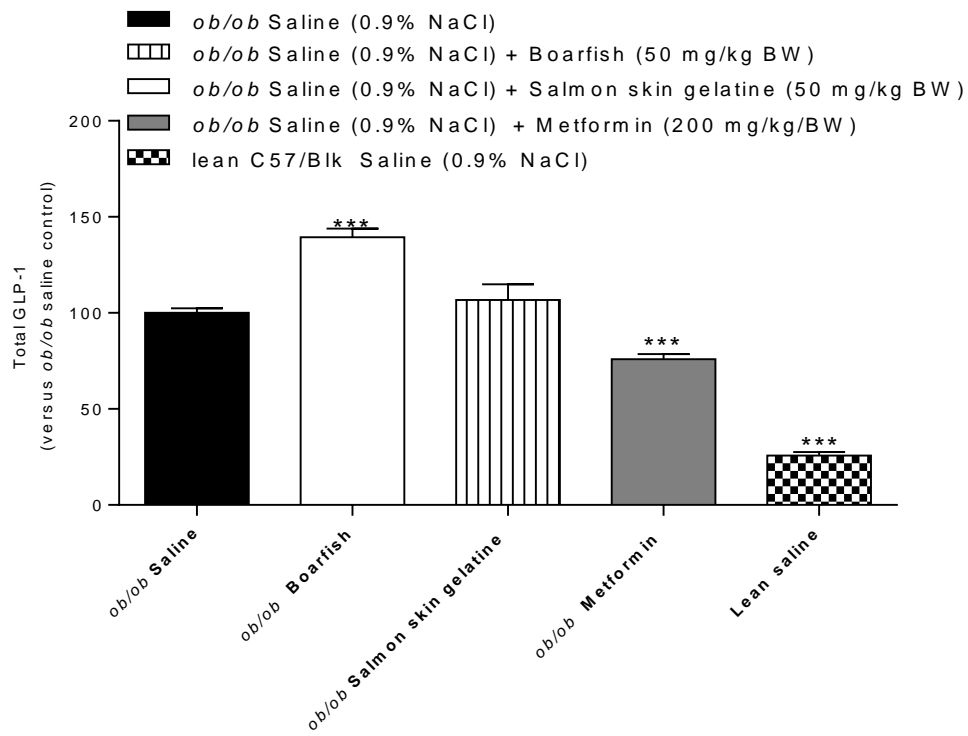


Fig 6.10. Effects of twice-daily administration of marine hydrolysates on terminal total plasma GLP-1 concentration. Values represent mean \pm SEM for 8 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus *ob/ob* saline control group.

Fig 6.11

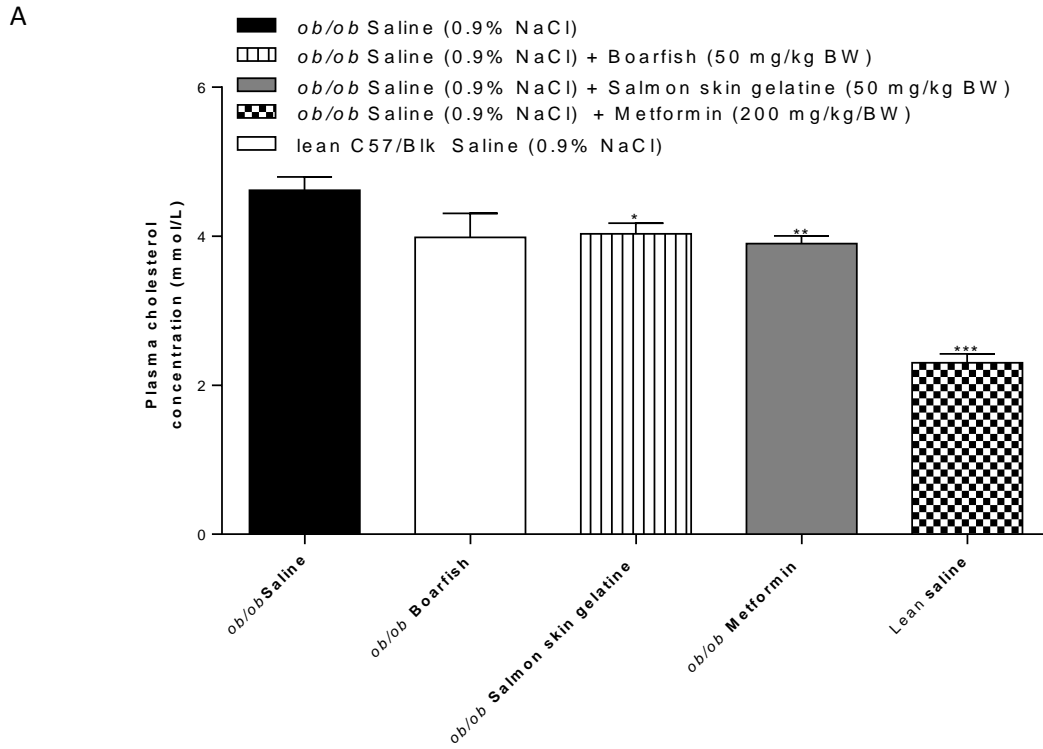


Fig 6.11 A. Effects of chronic administration of marine hydrolysates on terminal plasma cholesterol concentration. Values represent mean \pm SEM for 8 mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus *ob/ob* saline treated control group.

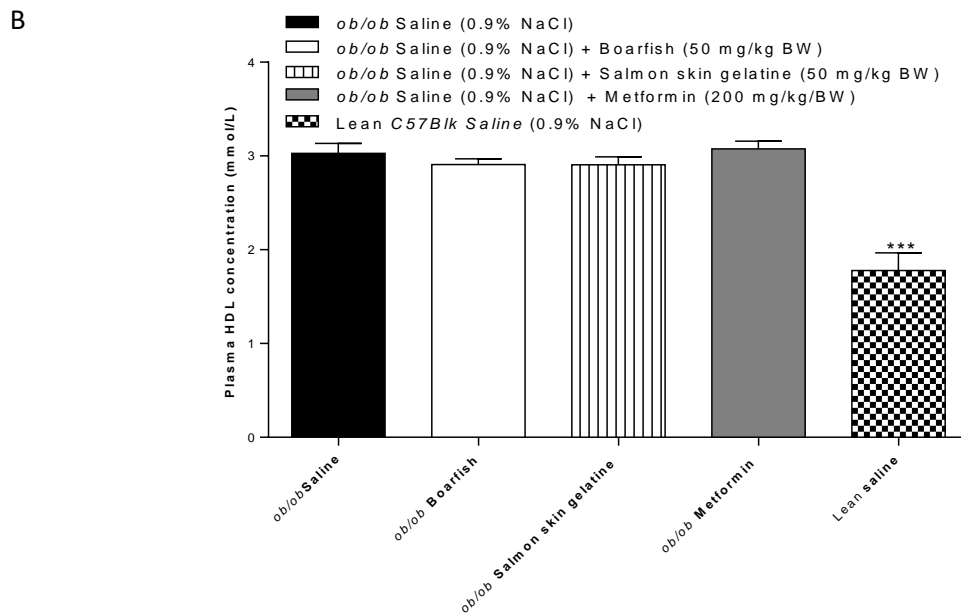


Fig 6.4.11 B. Effects of chronic administration of marine hydrolysates on terminal plasma HDL concentration. Values represent mean \pm SEM for 8 mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus *ob/ob* saline treated control group.

Fig 6.12

A

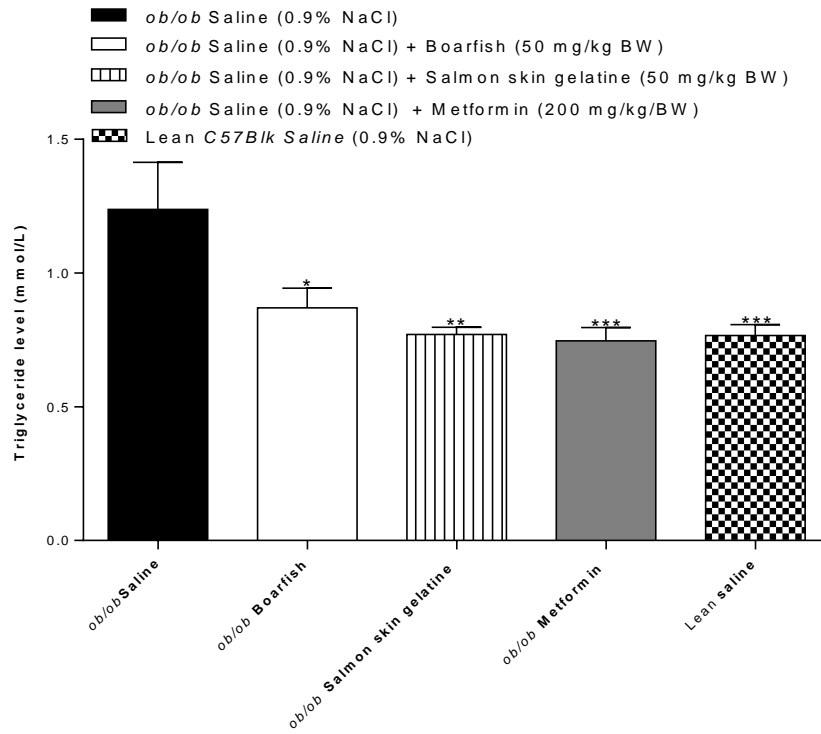


Fig 6.12 (A). Effects of chronic administration of marine hydrolysates on terminal plasma triglyceride (mmol/L) concentration. Values represent mean \pm SEM for 8 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus *ob/ob* saline control group.

B

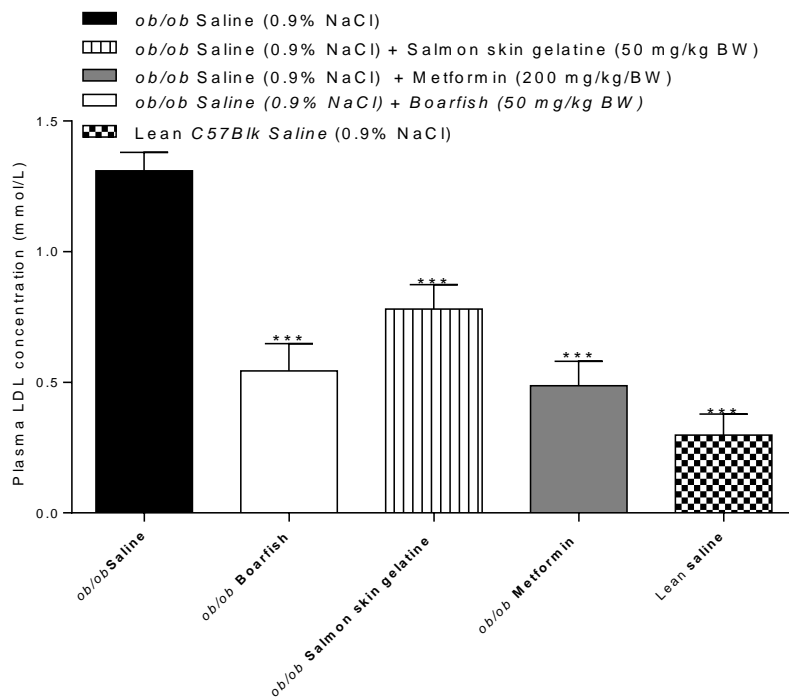
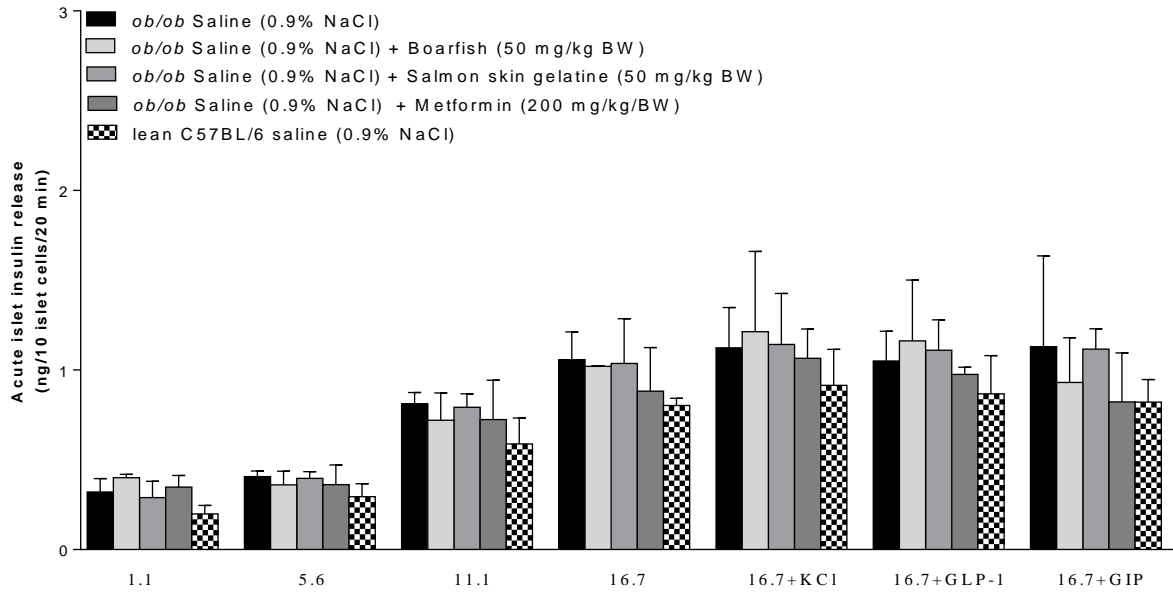


Fig 6.12 (B). Effects of chronic administration of marine hydrolysates on terminal plasma LDL (mmol/L) concentration. Values represent mean \pm SEM for 8 mice. *** $p < 0.001$ versus *ob/ob* saline control group.

Fig 6.13

A



B

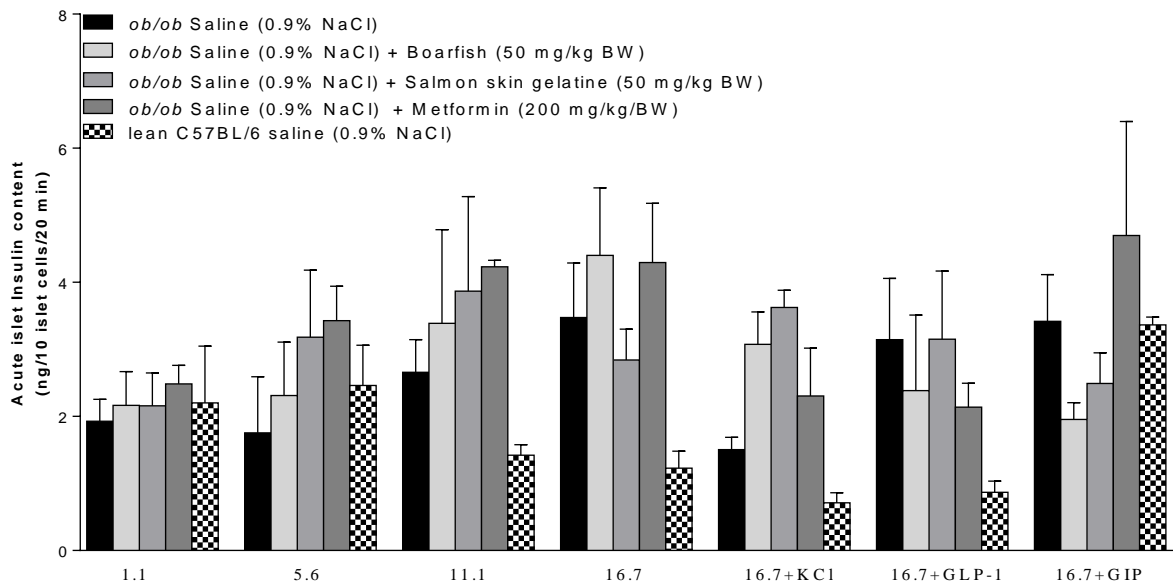


Fig 6.13. Effects of chronic administration of marine hydrolysates on isolated islet insulin secretion (A) and islet insulin content (B). Isolated islets were harvested from mice after the treatment period was passed (day 40). Islets were treated with various concentrations of glucose and known insulin secretagogues. A represents islets (10 islets per treatment per group) treated acutely for secretory activity. B represents the insulin content after acid ethanol extraction of islet tissue produced after the acute insulin secretory study was completed. The supernatant was removed, and pancreatic islets were lysed and insulin content (by RIA) measured in both supernatant and lysed samples.

Fig 6.14

A

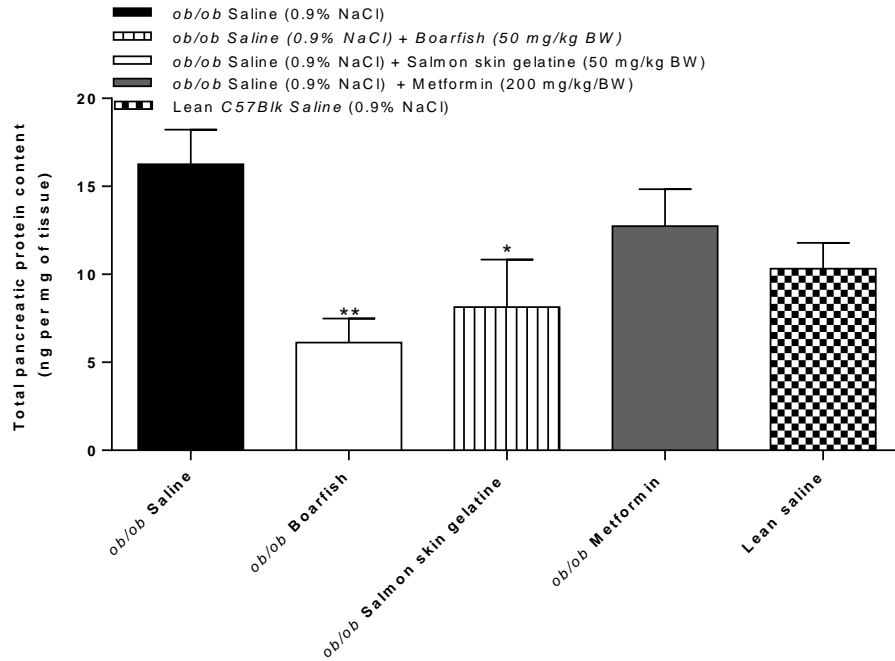


Fig 6.14 (A). Effects of chronic administration of marine hydrolysates on excised pancreata protein content. Whole pancreata were excised, weight and processed via acid ethanol extraction of tissue hormones. Hormone protein content was analysed using the Bradford protein assay. Values represent mean \pm SEM for 8 mice. * $p < 0.05$, ** $p < 0.01$, versus *ob/ob* saline

B

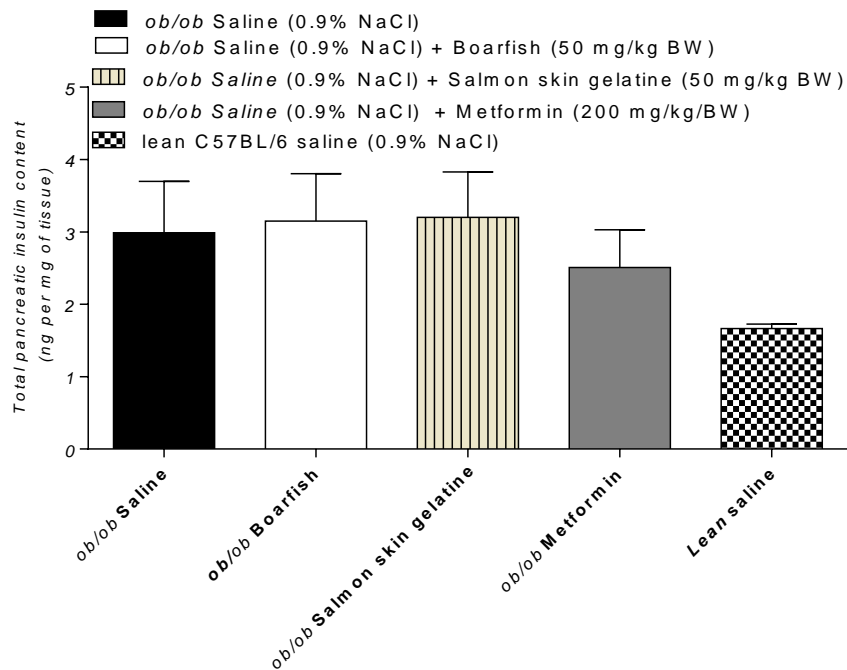


Fig 6.14 (B). Effects of chronic administration of marine hydrolysates on excised pancreati protein content. Whole pancreata were excised, weight and processed via acid ethanol extraction of tissue hormones. Hormones content was analysed using the Bradford protein assay. Values represent mean \pm SEM for 8 mice.

Fig 6.15

Group 1 – ob/ob saline treated control group

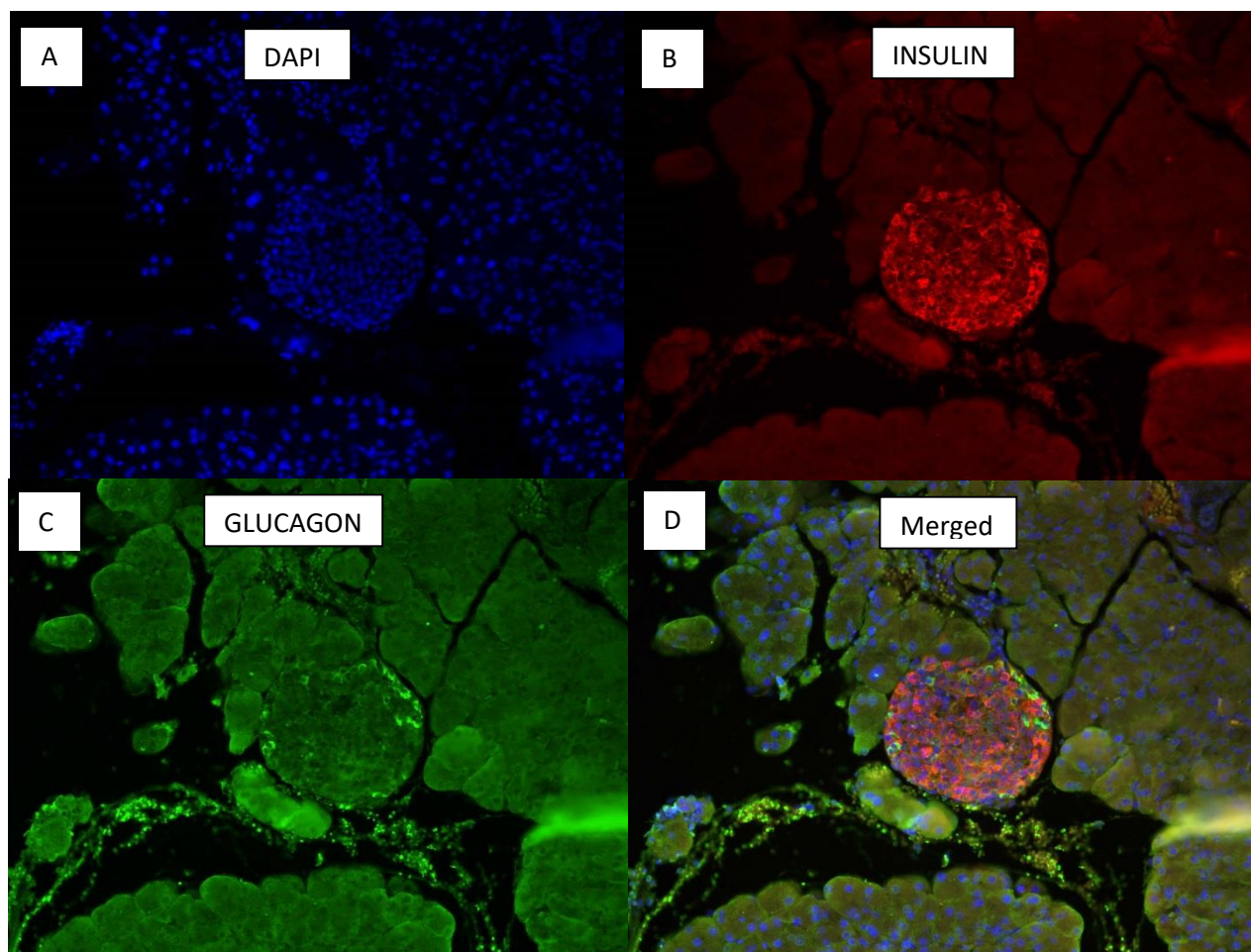


Fig 6.15. Effects of chronic administration of physiological saline on excised pancreatic islet morphology via immunohistochemistry analysis. Post-antigen retrieval slides were stained for **(A)** DAPI, **(B)** Insulin and **(C)** Glucagon. Greater than 50 islets were analysed per slide with analysis carried out using Image J.

Fig 6.16

Group 2 – ob/ob Boarfish protein hydrolysate treated group

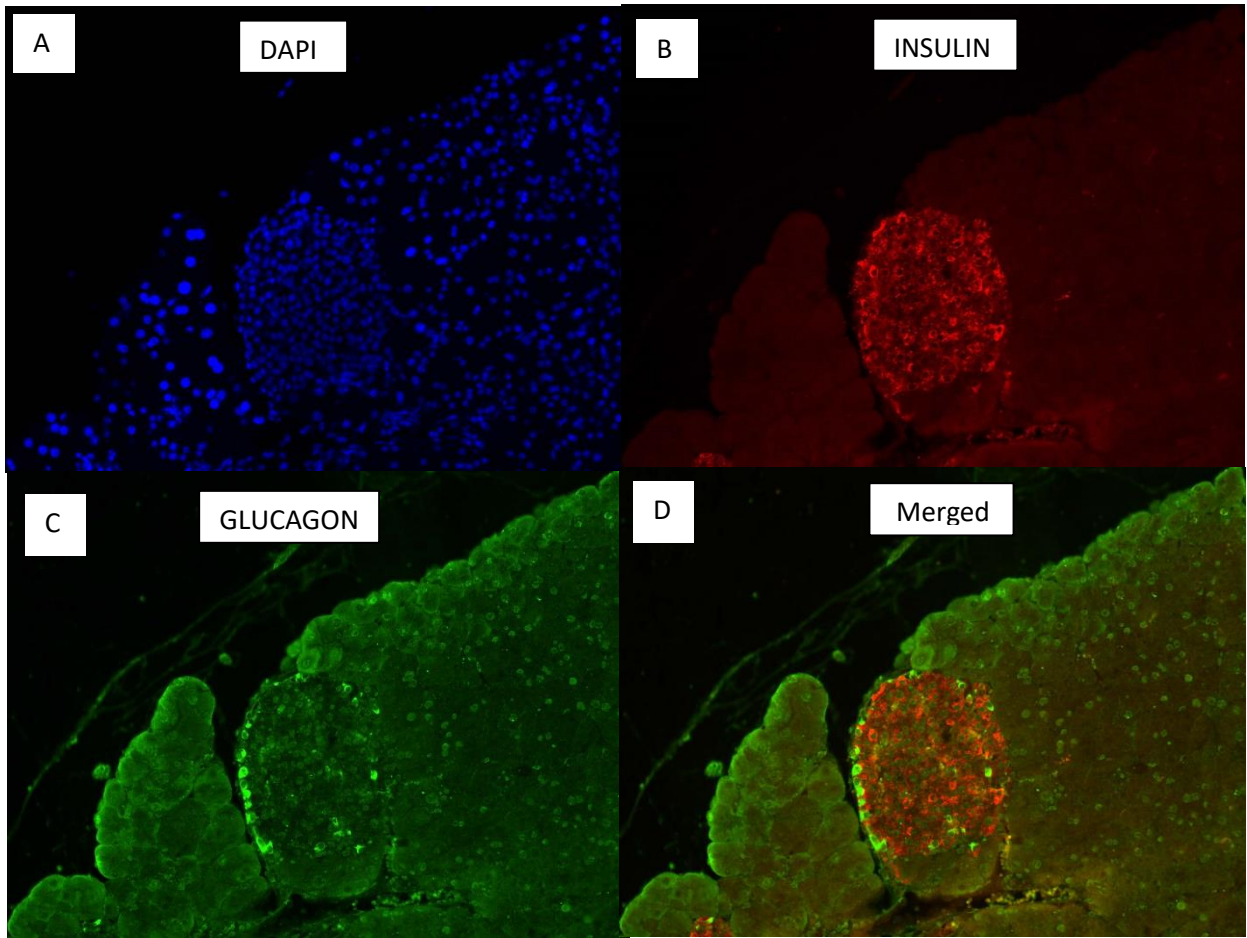


Fig 6.16. Effects of chronic administration of Boarfish protein hydrolysate on excised pancreatic islet morphology via immunohistochemistry analysis. Post-antigen retrieval slides were stained for **(A)** DAPI, **(B)** Insulin and **(C)** Glucagon. Greater than 50 islets were analysed per slide with analysis carried out using

Fig 6.17

Group 3 – ob/ob Salmon gelatine protein hydrolysate treated group

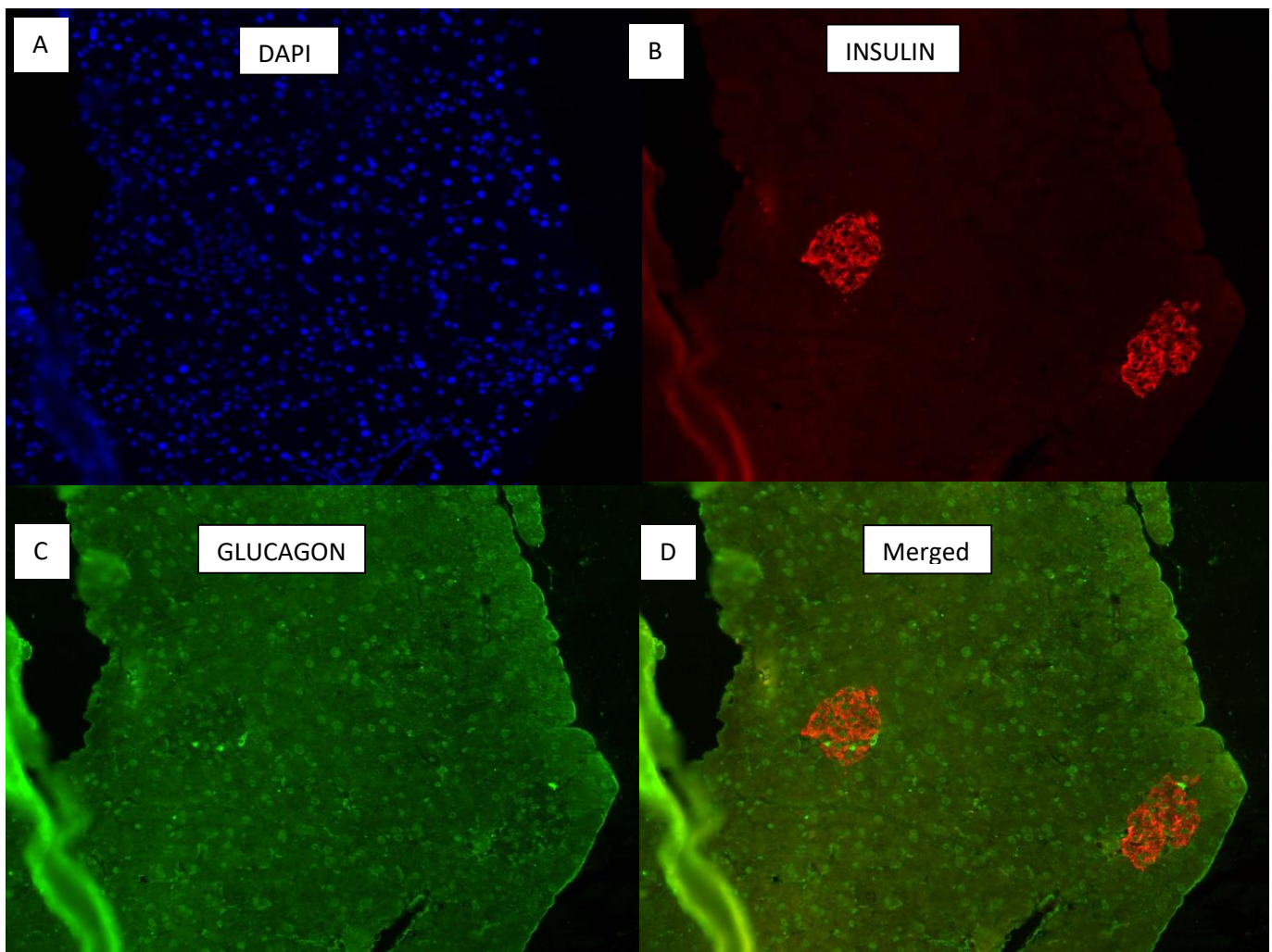


Fig 6.17. Effects of chronic administration of Salmon gelatine protein hydrolysate on excised pancreatic islet morphology via immunohistochemistry analysis. Post-antigen retrieval slides were stained for **(A)** DAPI, **(B)** Insulin and **(C)** Glucagon. Greater than 50 islets were analysed per slide with analysis carried

Fig 6.18

Group 4 – ob/ob Metformin treated group

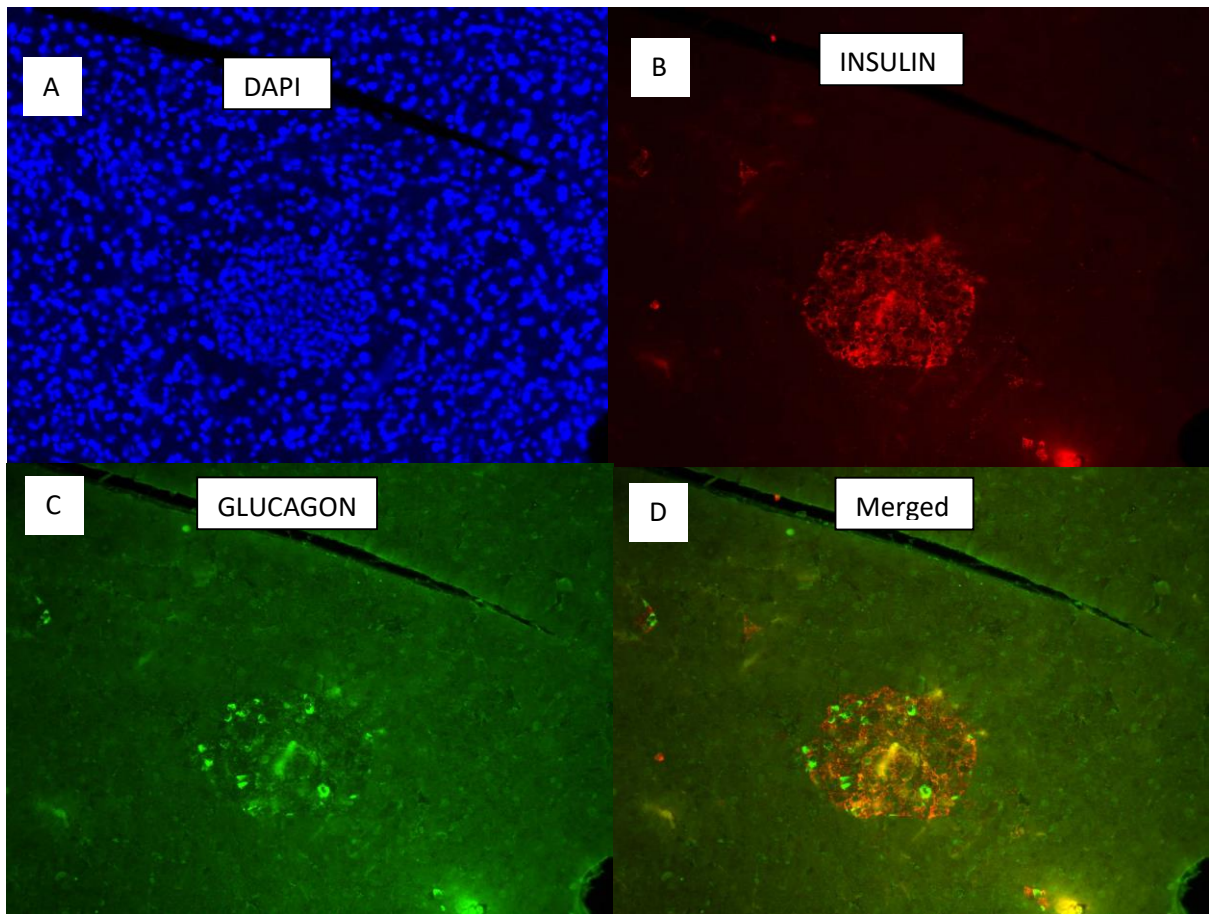


Fig 6.18. Effects of chronic administration of Metformin on excised pancreatic islet morphology via immunohistochemistry analysis. Post-antigen retrieval slides were stained for **(A)** DAPI, **(B)** Insulin and **(C)** Glucagon. Greater than 50 islets were analysed per slide with analysis carried out using Image J.

Fig 6.19

A

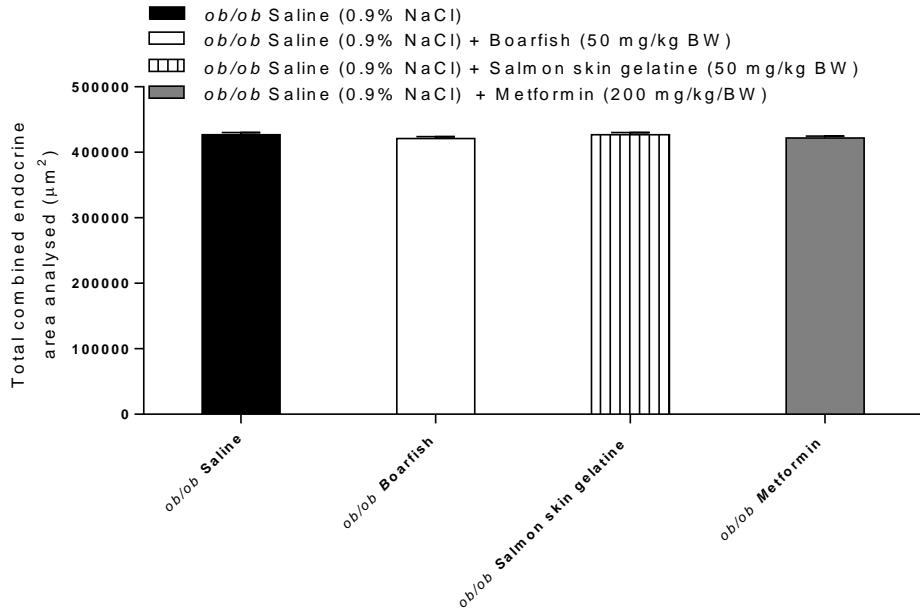


Fig 6.19 (A). Effects of twice-daily administration of marine hydrolysates on pancreata morphology. Graph represents total tissue section (μm^2) Values represent mean \pm SEM for 8 mice.

B

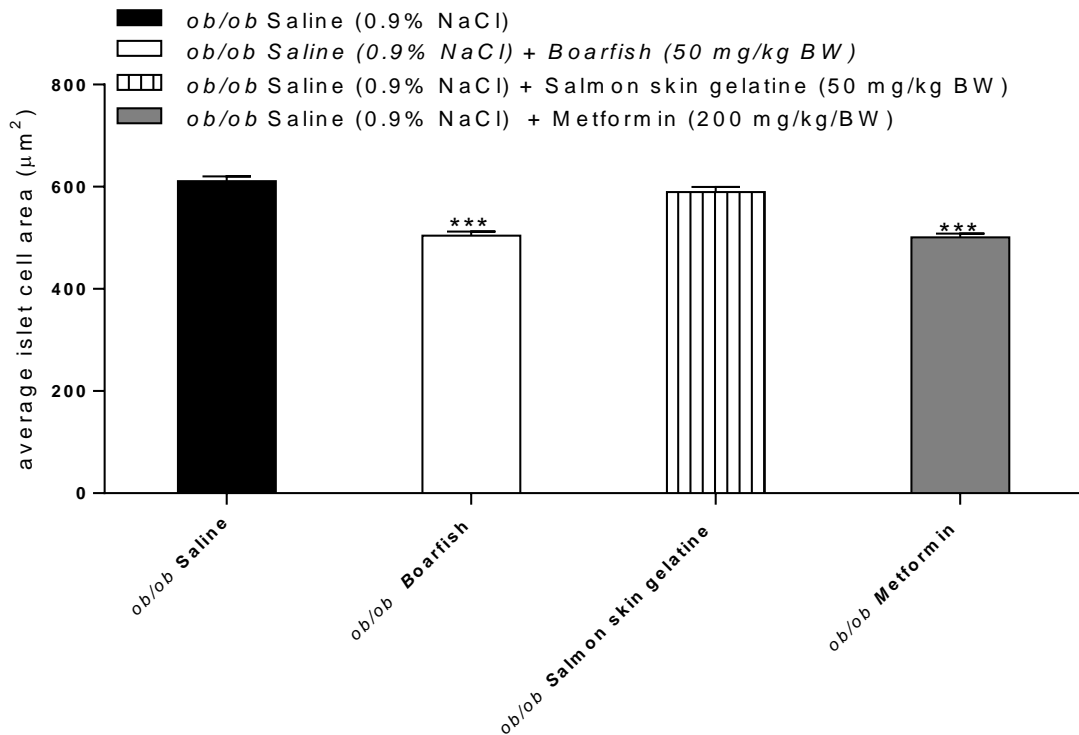


Fig 6.19 (B). Effects of twice-daily administration of marine hydrolysates on pancreatic islet morphology. Graph represents average islet cell area (μm^2). Values represent mean \pm SEM for 8 mice. *** $p < 0.01$, versus *ob/ob* saline treated control group.

Fig 6.20

A

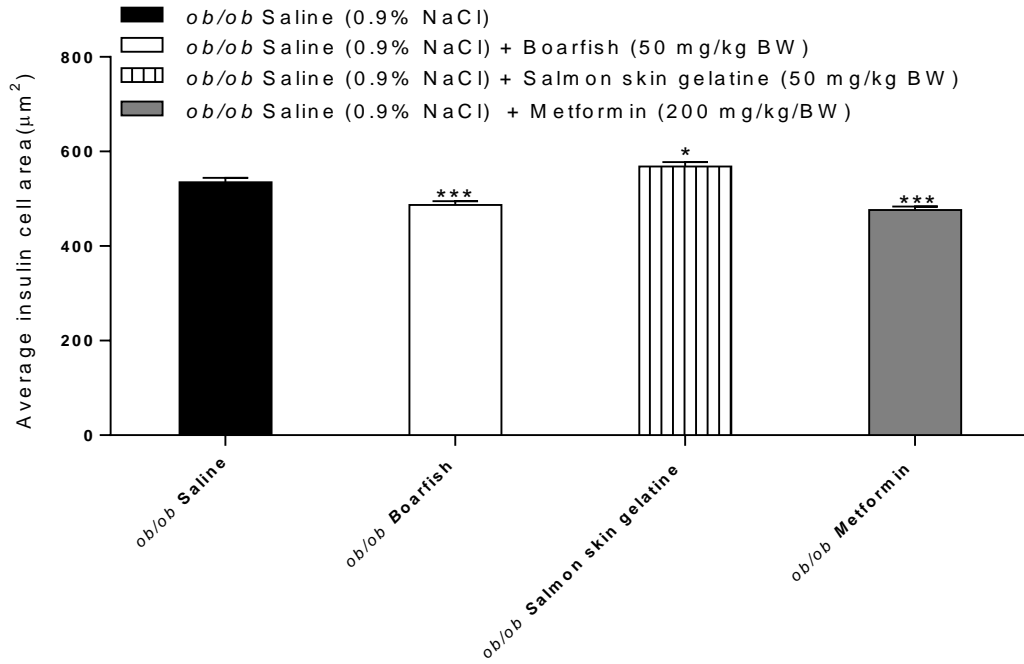


Fig 6.20 (A). Effects of twice-daily administration of marine hydrolysates on pancreatic islet morphology. Graph represents average insulin/beta cell area (µm²). Values represent mean ± SEM for 8 mice. *p<0.05, ***p<0.01, versus *ob/ob* saline control treated group,

B

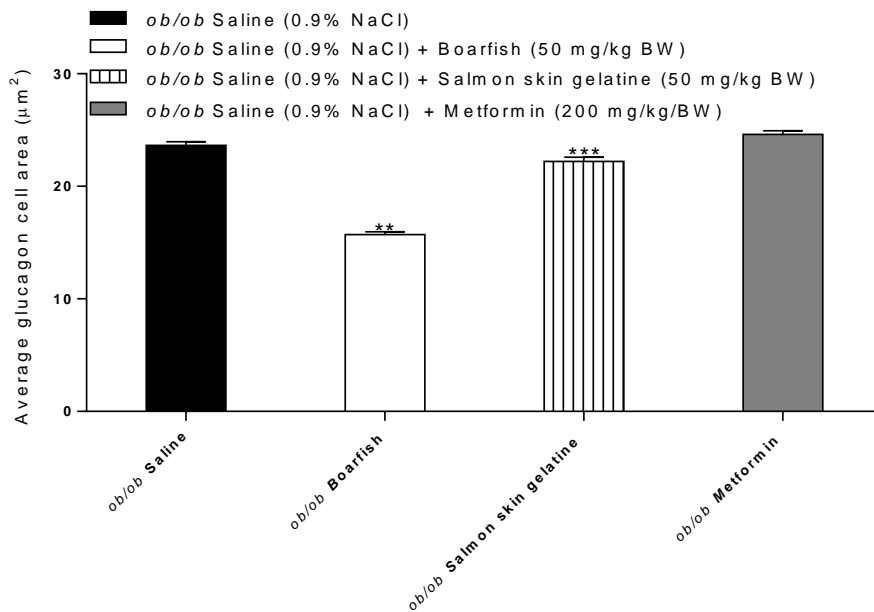


Fig 6.20 (B). Effects of twice-daily administration of marine hydrolysates on pancreatic islet morphology. Graph represents average glucagon/alpha cell area (µm²). Values represent mean ± SEM for 8 mice. **p<0.01, ***p<0.001 versus *ob/ob* saline control treated group.

Fig 6.21

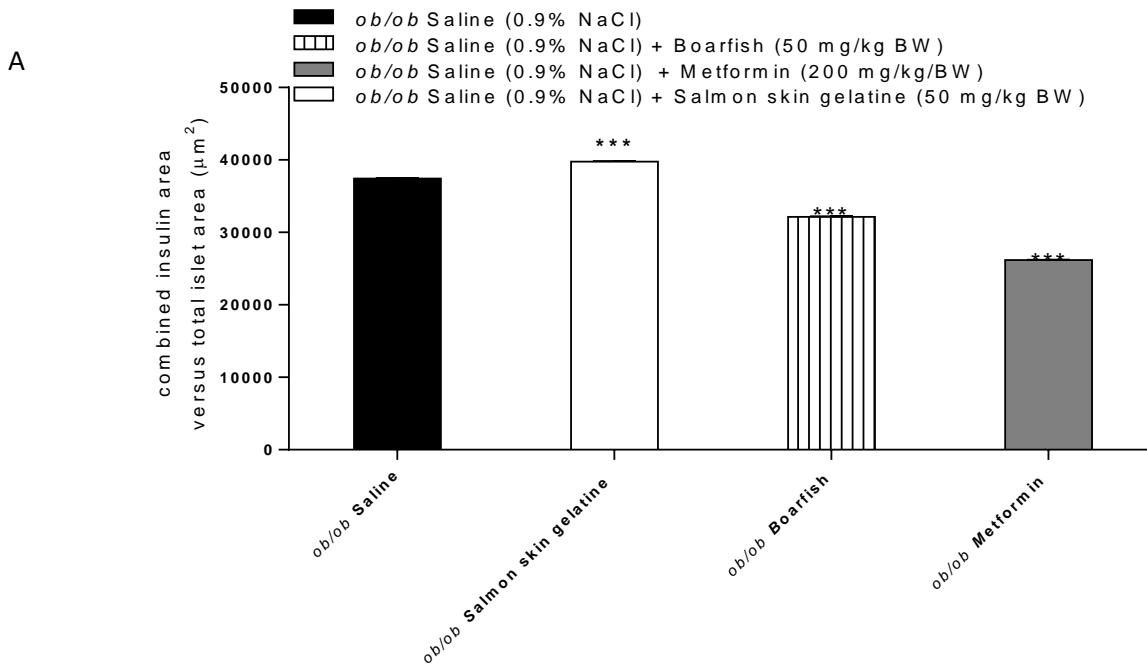


Fig 6.21 (A). Effects of twice-daily administration of marine hydrolysates on pancreatic islet morphology. Graph represents total insulin stained cell area for all islets (μm^2). Values represent mean \pm SEM for 8 mice. *** $p < 0.001$, versus *ob/ob* saline treated control group.

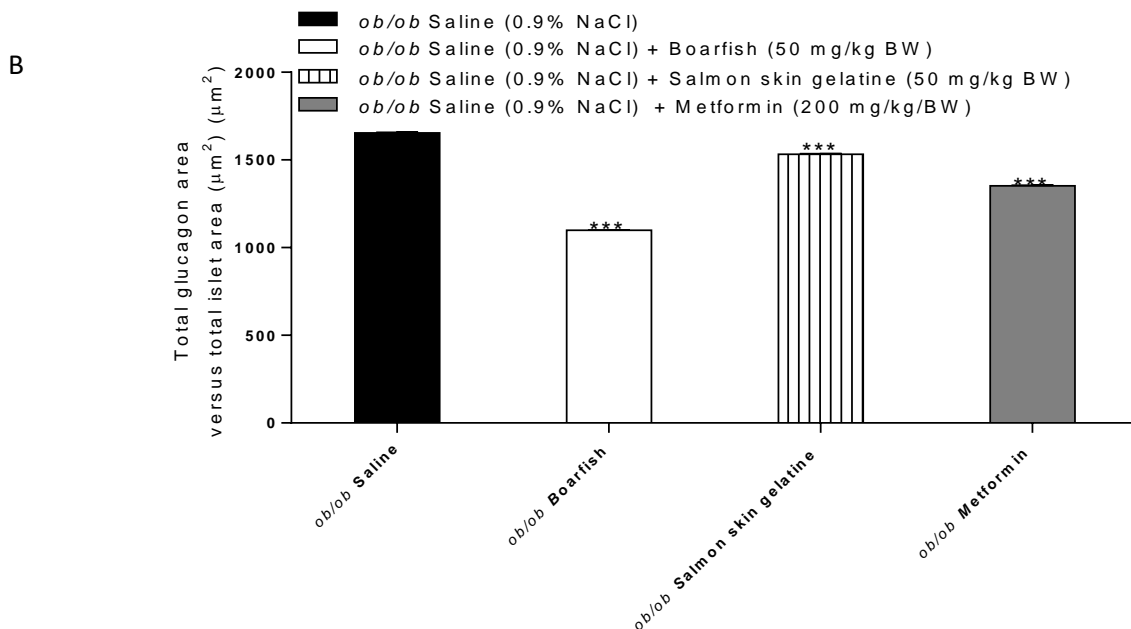


Fig 6.21 (B). Effects of twice-daily administration of marine hydrolysates on pancreatic islet morphology. Graph represents total glucagon stained area for all islets imaged (μm^2). Values represent mean \pm SEM for 8 mice, *** $p < 0.001$, versus *ob/ob* saline control group.

Fig 6.22

A

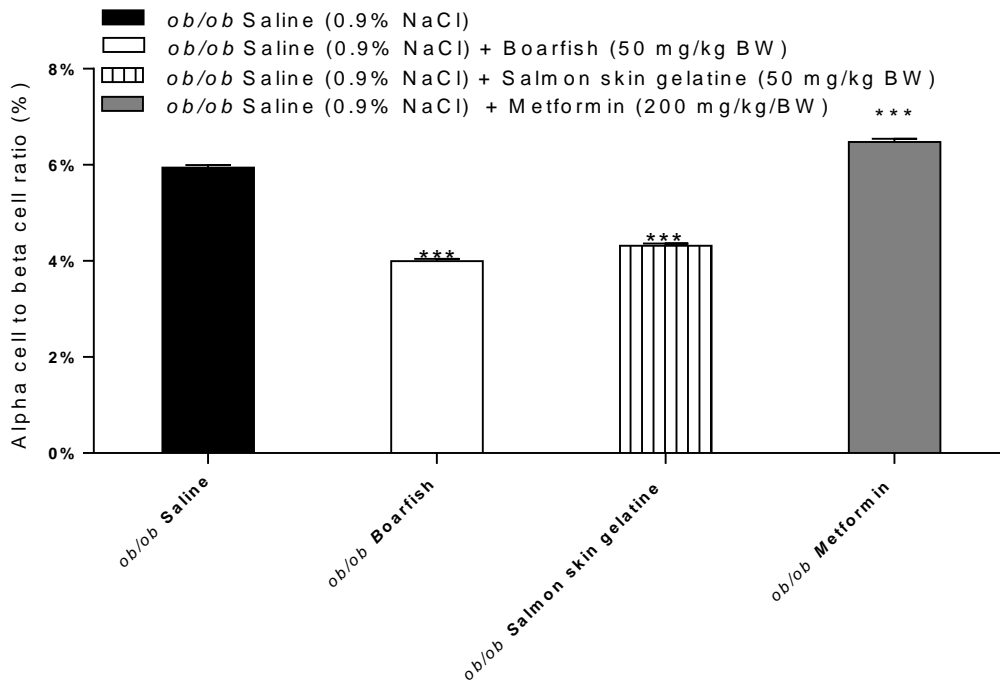


Fig 6.22 (A). Effects of twice-daily administration of marine hydrolysates on pancreata morphology. Graph represents alpha to beta cell ratio (%). Values represent mean \pm SEM for 8 mice. * $p < 0.05$, *** $p < 0.01$, versus *ob/ob* saline control group.

Anthropometric analysis and bone parameter (DEXA)

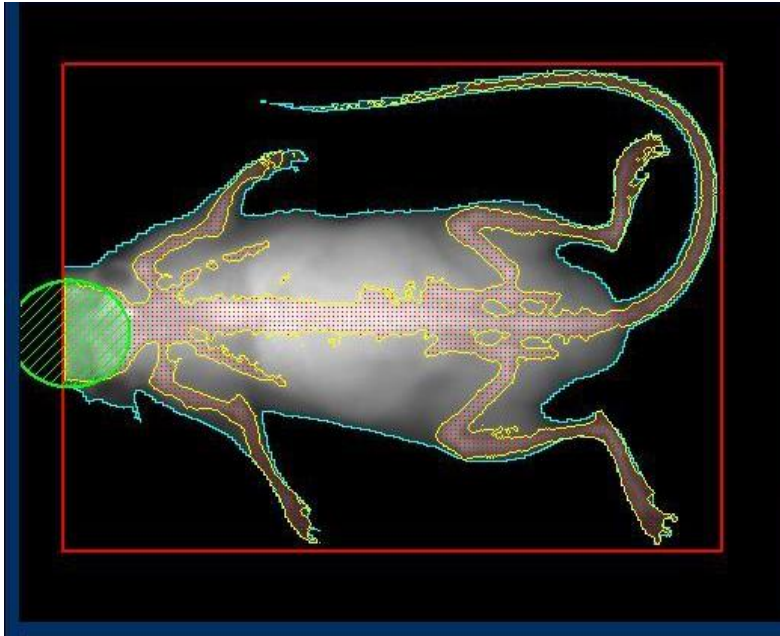


Fig 6.23

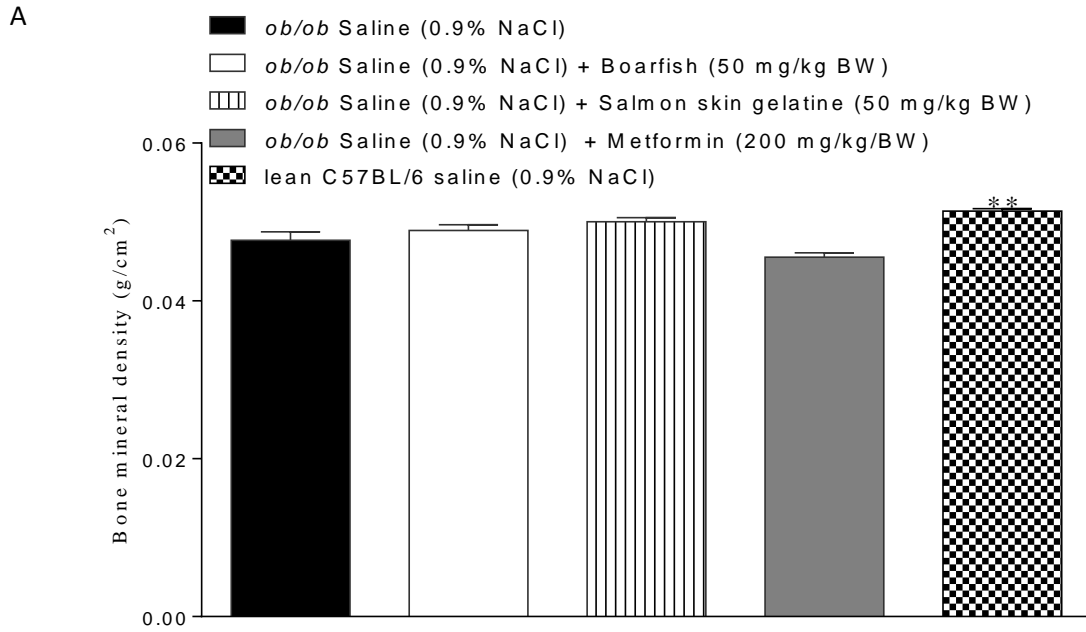


Fig 6.23 (A). Effects of twice-daily administration of marine hydrolysates on bone mineral density analysis assessed via PIXImus DEXA. Graph represents total body bone mineral density (BMD g/cm²). Values represent mean \pm SEM for 8 mice. ** $p < 0.01$, versus *ob/ob* saline treated control

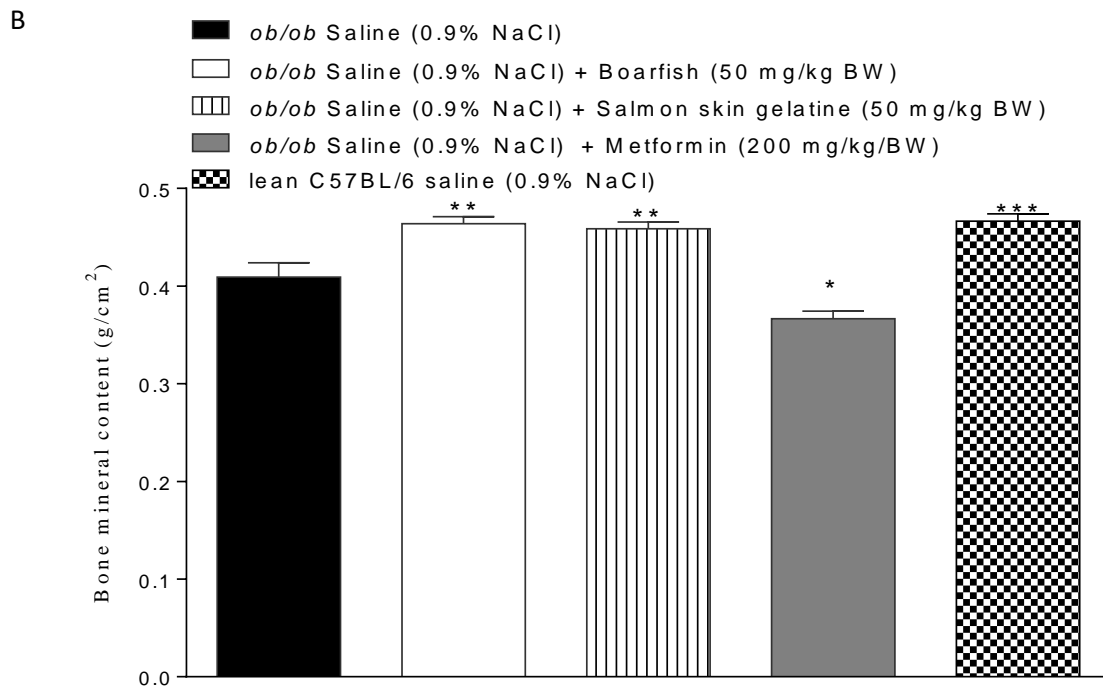


Fig 6.23 (B). Effects of twice-daily administration of marine hydrolysates on bone mineral density as assessed via PIXImus DEXA. Graph represents total body bone mineral content (BMD g/cm²). Values represent mean \pm SEM for 8 mice. * $p < 0.05$, ** $p < 0.01$, versus *ob/ob* saline control group.

Fig 6.24

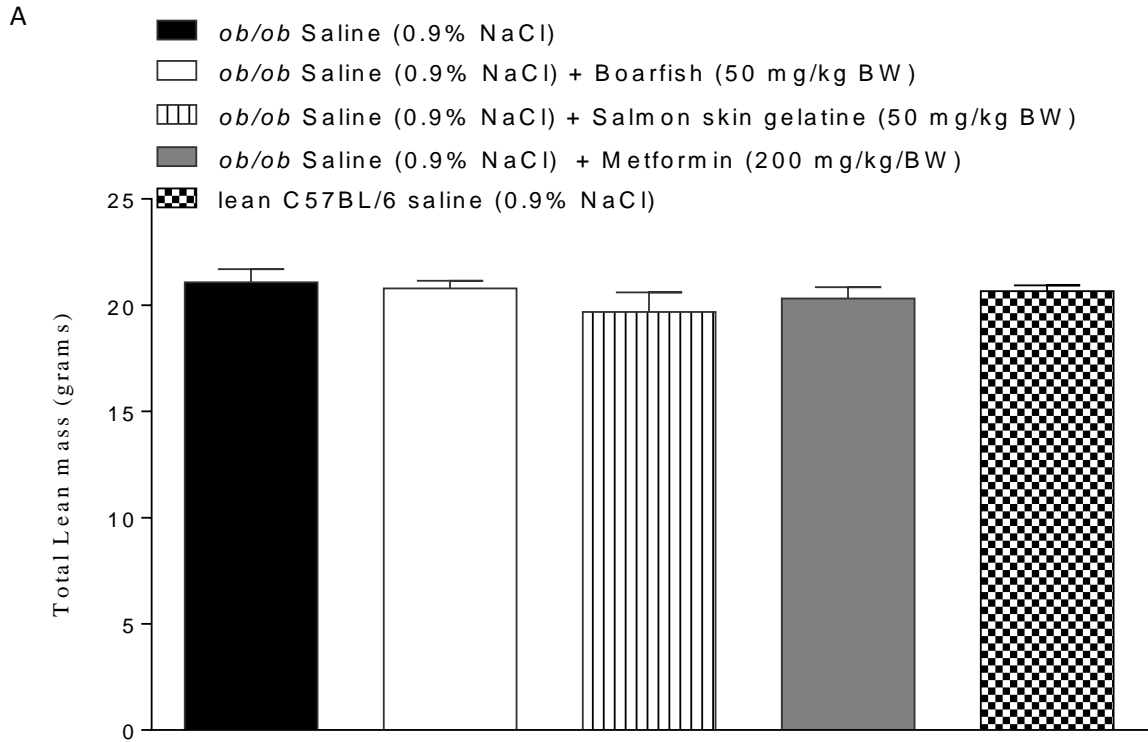


Fig 6.24 (A). Effects of twice-daily administration of marine hydrolysates on total lean mass as assessed via PIXImus DEXA. Graph represents total body lean mass (grams) Values represent mean \pm SEM for 8 mice.

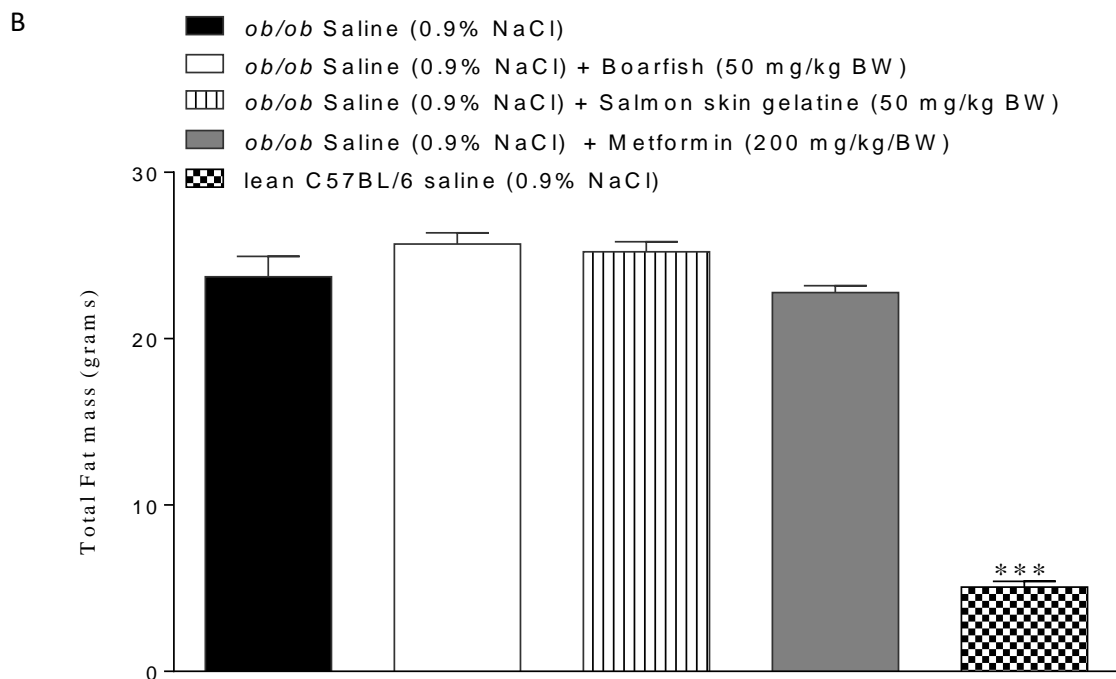
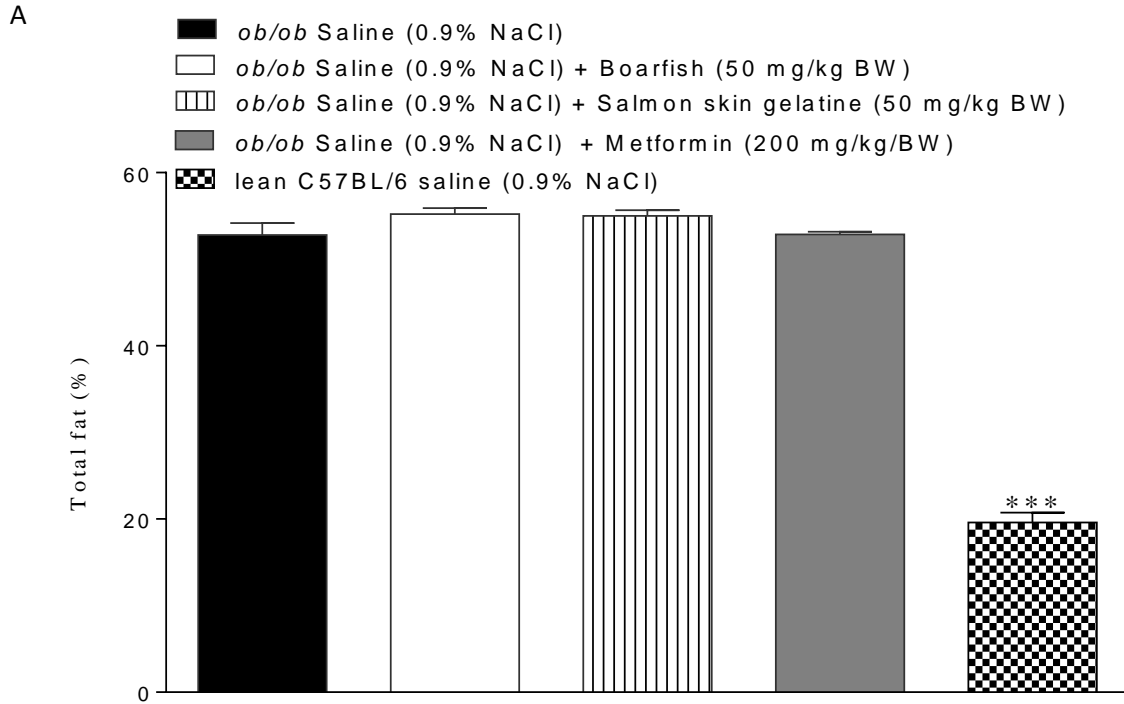


Fig 6.24 (B). Effects of twice-daily administration of marine hydrolysates on total fat mass as assessed via PIXImus DEXA. Graph represents total body fat mass (grams) Values represent mean \pm SEM for 8 mice. *** $p < 0.001$ versus *ob/ob* saline control group.

Fig 6.25



B Fig 6.25 (A). Effects of twice-daily administration of marine hydrolysates on % body fat mass as assessed via PIXImus DEXA. Graph represents total body fat (%) Values represent mean \pm SEM for 8 mice. *** $p < 0.001$ versus *ob/ob* saline treated control group.

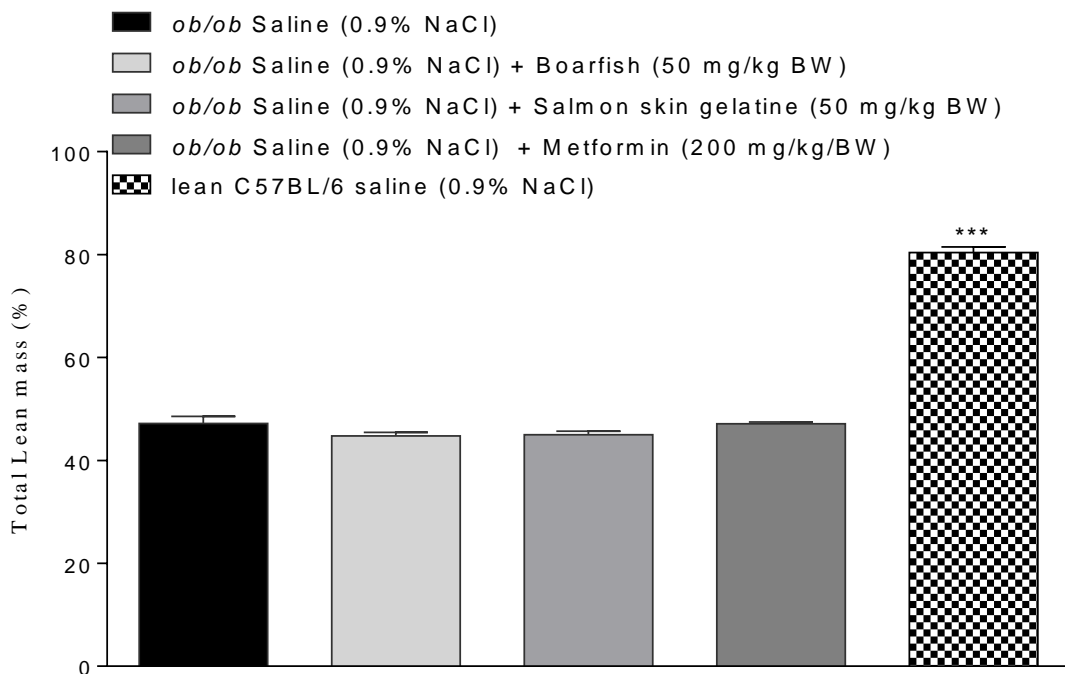


Fig 6.25 (B). Effects of twice-daily administration of marine hydrolysates on anthropometric analysis assessed via PIXImus DEXA. Graph represents total body lean mass percentage. Values represent mean \pm SEM for 8 mice. *** $p < 0.001$ versus *ob/ob* saline control group.

Fig 6.26

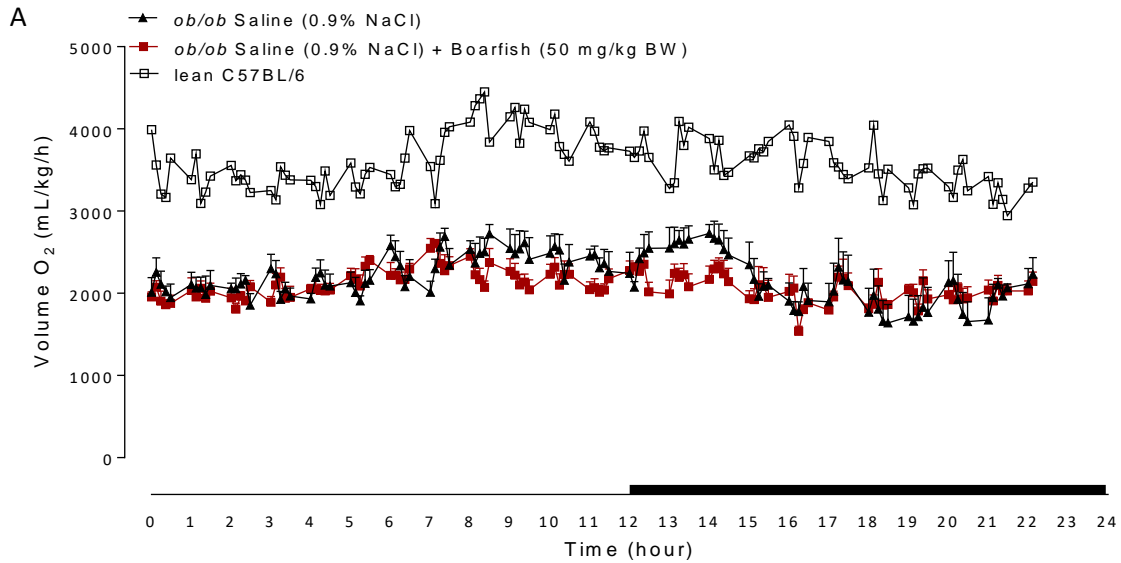


Fig 6.26 (A). Energy expenditure after twice daily boarfish hydrolysate administration in *ob/ob* mice. The mice were single housed and energy expenditure was normalized for 12 h prior to 12 more consecutive analysis h ($n = 6$). Gas exchange (Volume O_2) was measured at post-27 days of treatment. The data are expressed as mean \pm SEM. Black bar represents the dark room cycle with area without representing light room cycle (12 h:12 h).

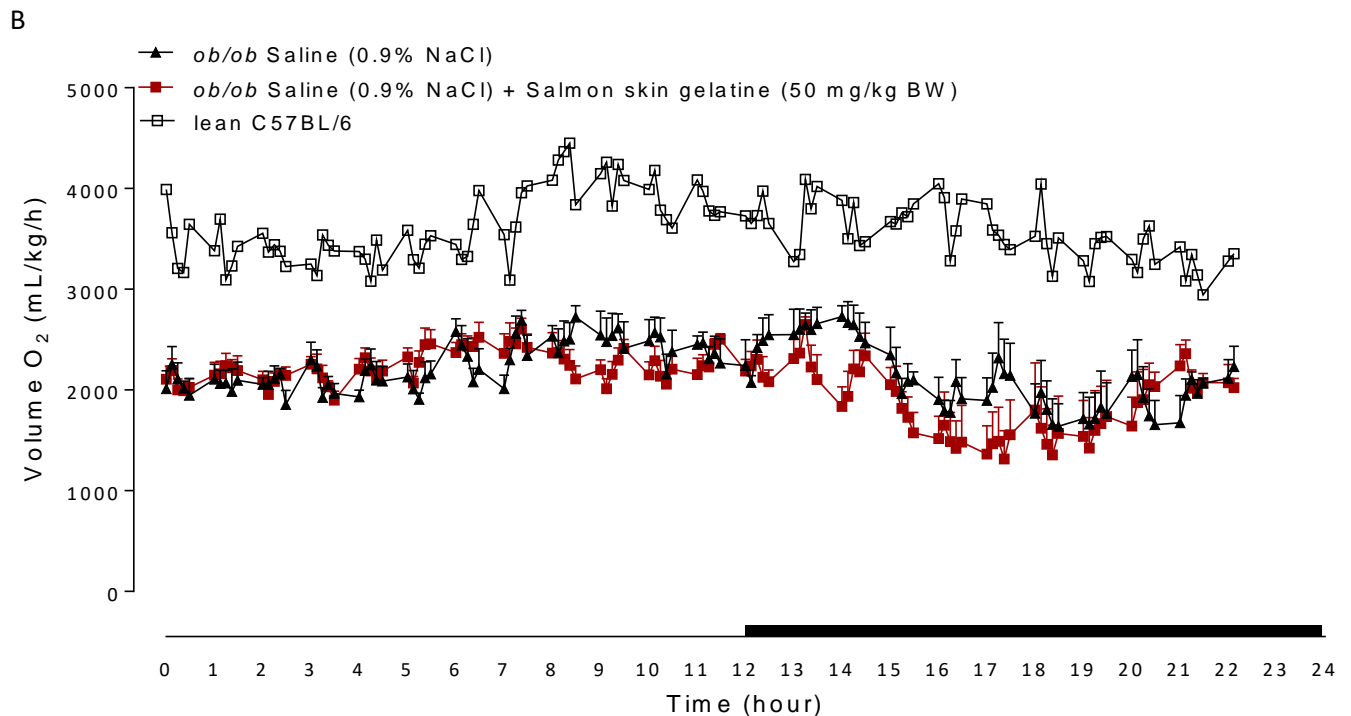


Fig 6.26 (B). Energy expenditure after chronic salmon gelatine hydrolysate administration in *ob/ob* mice. The mice were single housed and energy expenditure was normalized for 12 h prior to 12 more consecutive analysis h ($n = 6$). Gas exchange (Volume O_2) was measured at post-27 days of treatment. The data are expressed as mean \pm SEM. Black bar represents the dark room cycle with area without representing light room cycle (12 h:12 h).

Fig 6.27

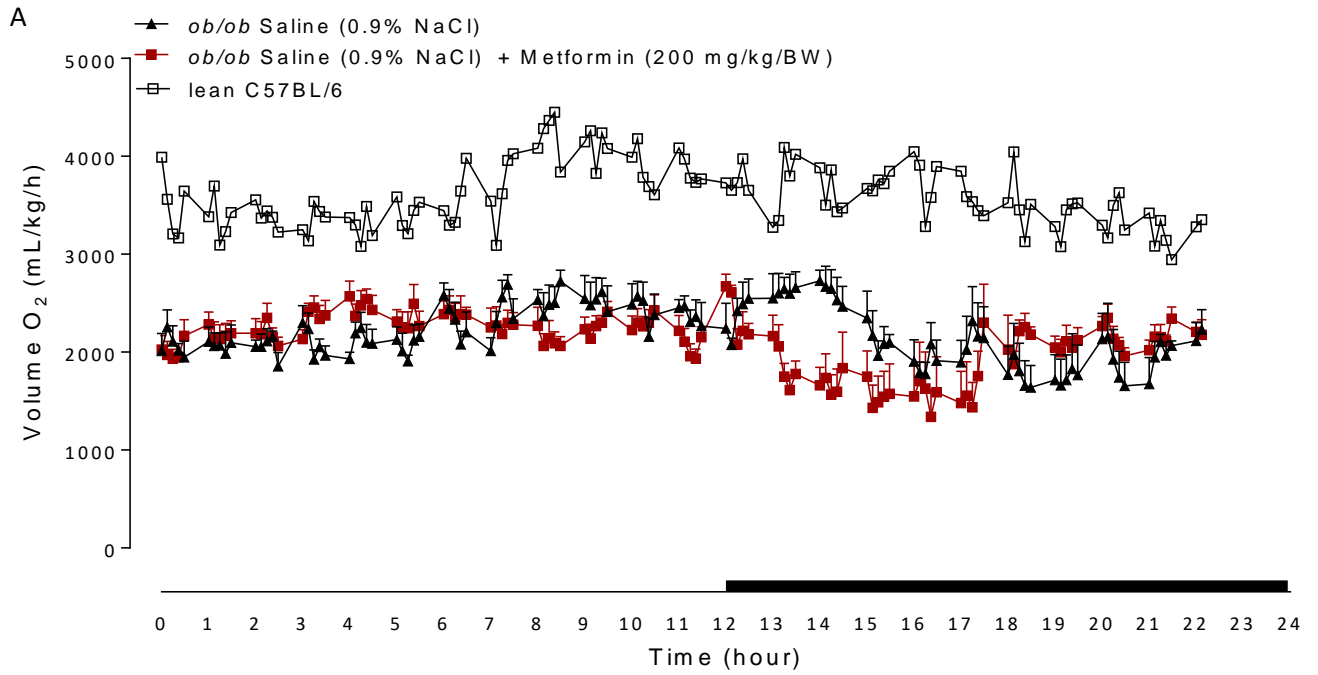


Fig 6.27 (A). Energy expenditure after twice-daily metformin administration in *ob/ob* mice. The mice were single housed and energy expenditure was normalized for 12 hours prior to 12 more consecutive analysis hours ($n = 6$). Gas exchange (Volume O₂) was measured at post-27 days of treatment. The data are expressed as mean \pm SEM. Black bar represents the dark room cycle with area without representing light

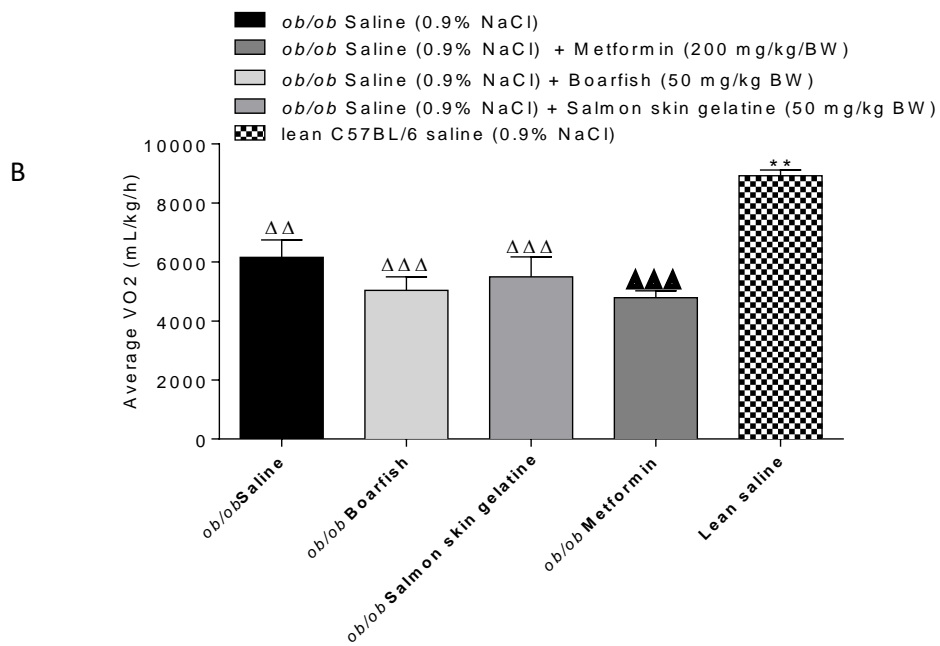


Fig 6.27 (B). Mean energy expenditure post study in ob/ob mice. The mice were single housed and energy expenditure was normalized for 12 hours prior to 12 more consecutive analysis hours (n = 6). Graph represents average VO₂ (Volume O₂) exchanged at post-27 days of treatment. The data are expressed as mean ± SEM.

Fig 6.28

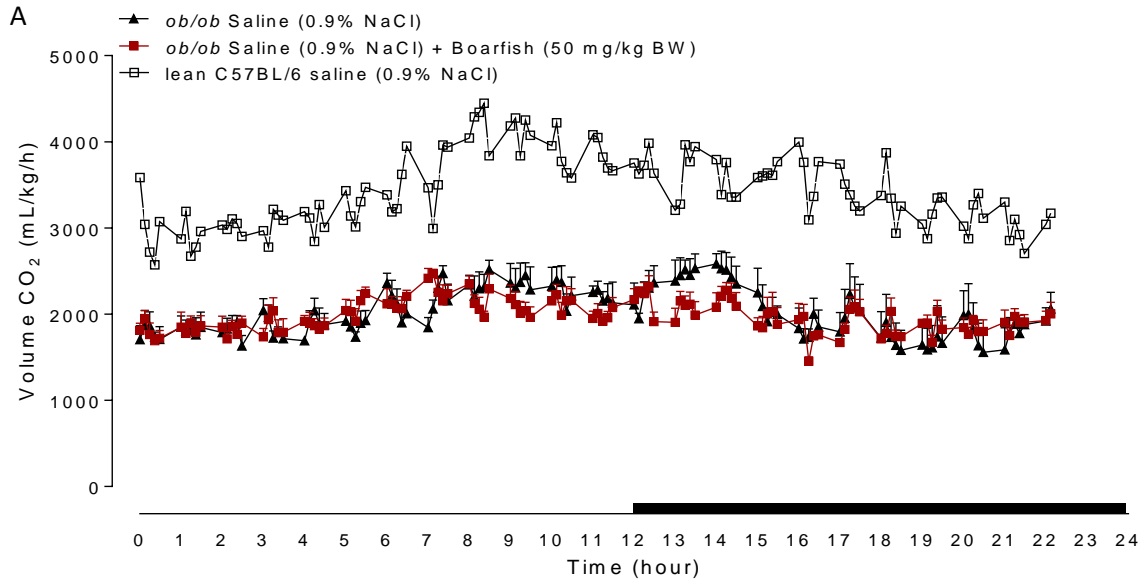


Fig 6.28 (A). Energy expenditure after twice-daily boarfish administration in *ob/ob* mice. The mice were single housed and energy expenditure was normalized for 12 h prior to 12 more consecutive analysis hours ($n = 6$). Gas exchange (Volume CO₂) was measured at post-27 days of treatment. The data are expressed as mean \pm SEM. Black bar represents the dark room cycle with area without representing light room cycle

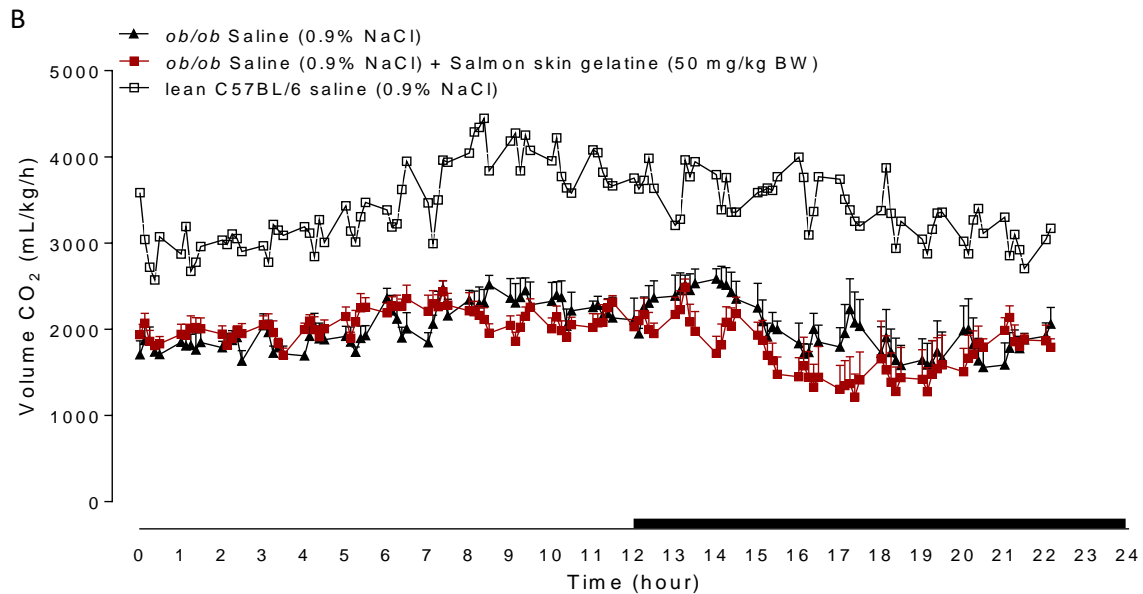


Fig 6.28 (B). Energy expenditure after twice-daily salmon skin gelatine administration in *ob/ob* mice. The mice were single housed and energy expenditure was normalized for 12 h prior to 12 more consecutive analysis hours ($n = 6$). Gas exchange (Volume CO₂) was measured at post-27 days of treatment. The data are expressed as mean \pm SEM. Black bar represents the dark room cycle with area without representing

Fig 6.29

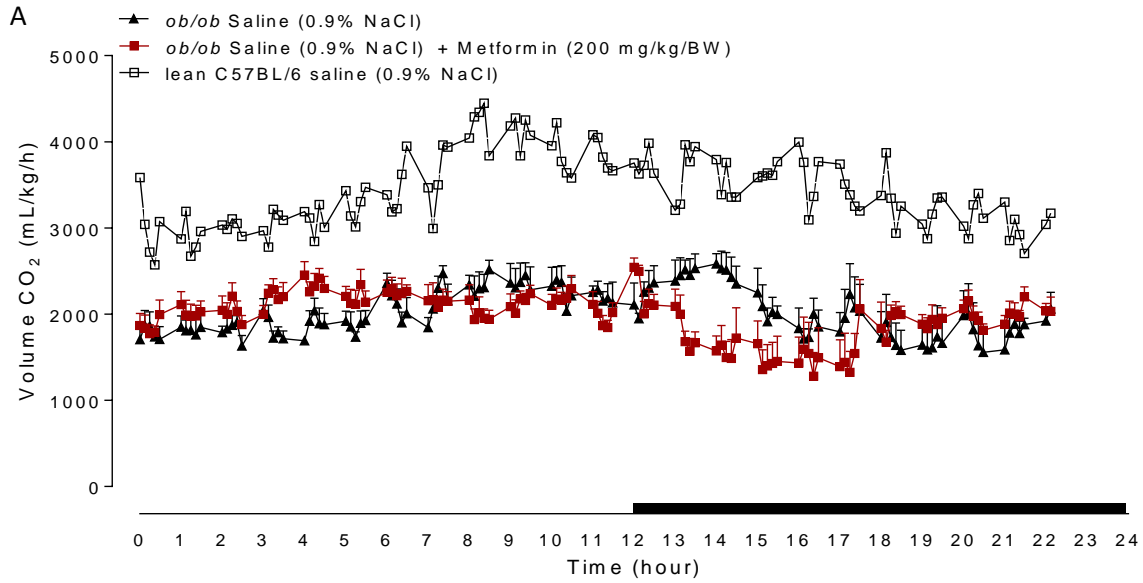


Fig 6.29 (A). Energy expenditure after twice-daily metformin administration in *ob/ob* mice. The mice were single housed and energy expenditure was normalized for 12 hours prior to 12 more consecutive analysis hours (n = 6). Gas exchange (Volume CO₂) was measured at post-27 days of treatment. The data are expressed as mean ± SEM. Black bar represents the dark room cycle with area without representing light

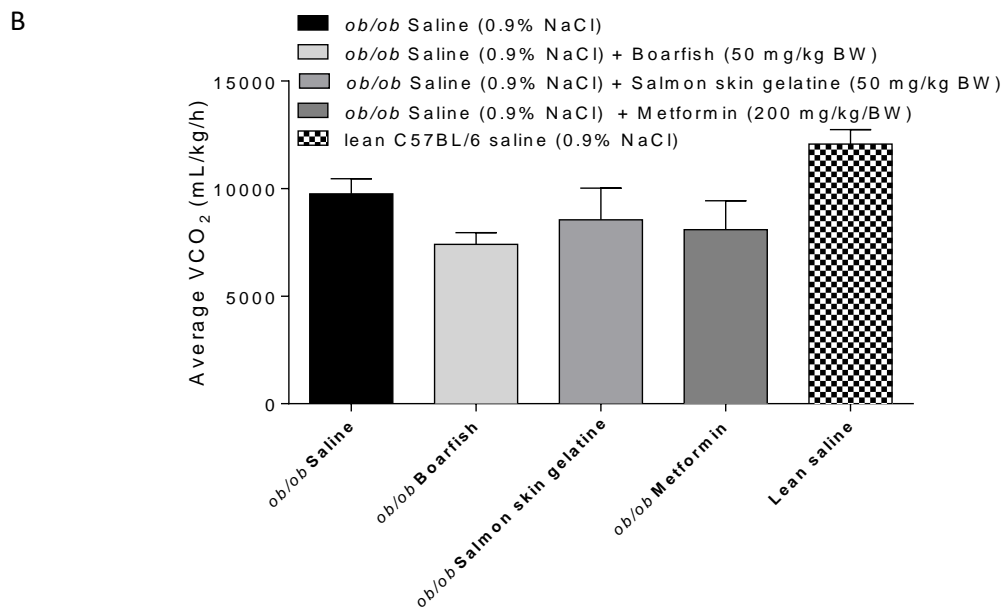


Fig 6.29 (B). Mean energy expenditure post study in *ob/ob* mice. The mice were single housed and energy expenditure was normalized for 12 hours prior to 12 more consecutive analysis hours (n = 6). Graph represents average VCO₂ (Volume VCO₂) exchanged at post-27 days of treatment. The data are expressed as mean ± SEM.

Fig 6.30

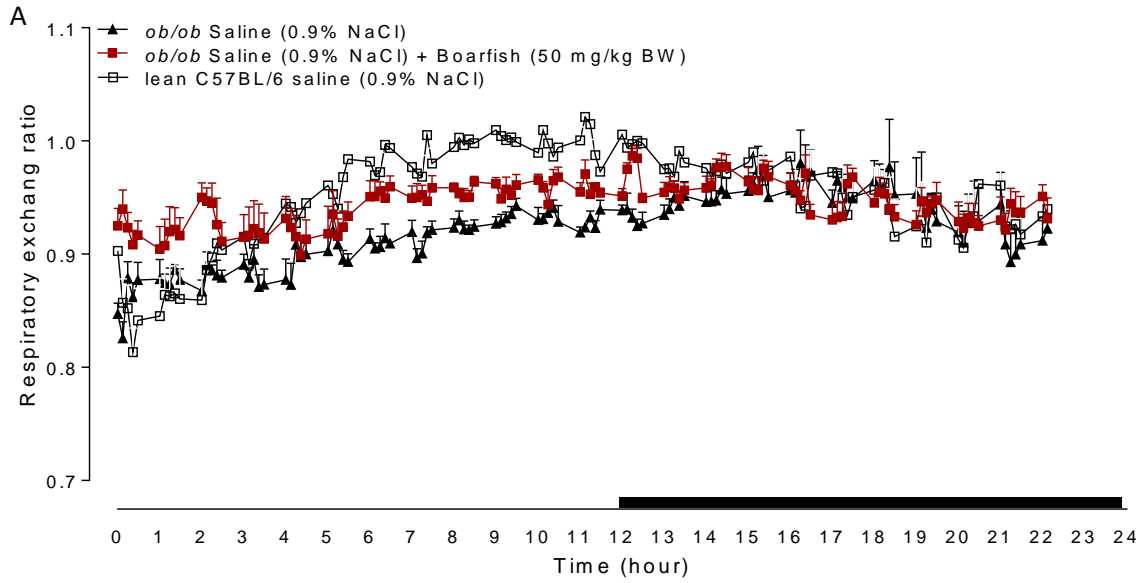


Fig 6.30 (A). Respiratory exchange ratio after twice-daily boarfish administration in *ob/ob* mice. The mice were single housed and energy expenditure was normalized for 12 h prior to 12 more consecutive analysis h (n = 6). Respiratory exchange (RER) was measured at post-27 days of treatment. The data are expressed as mean \pm SEM. Black bar represents the dark room cycle with area without representing light room cycle

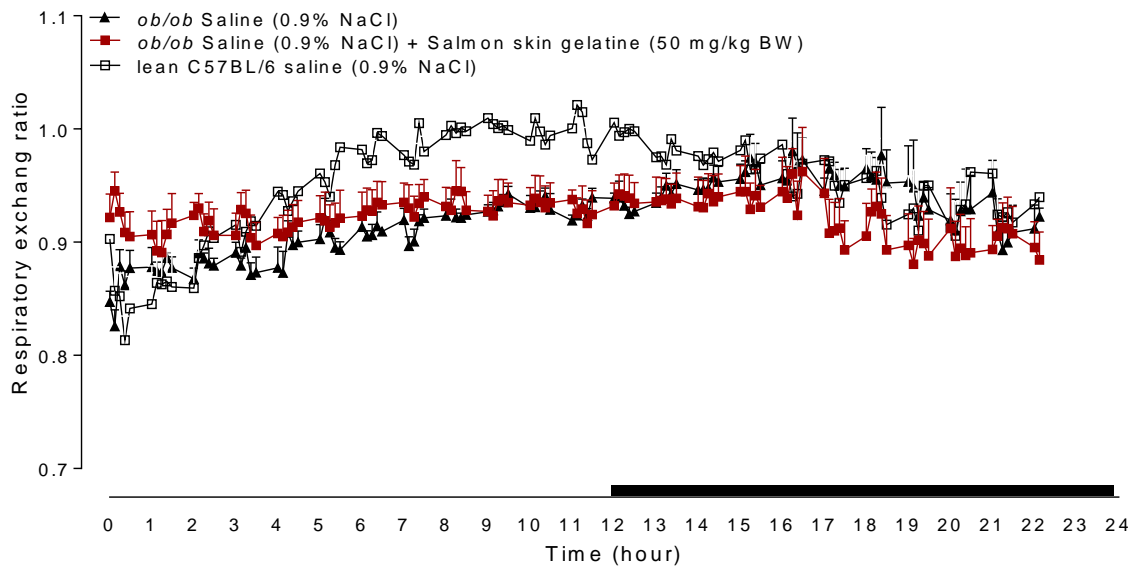


Fig 6.30 (B). Respiratory exchange ratio after twice-daily salmon skin gelatine administration in *ob/ob* mice. The mice were single housed and energy expenditure was normalized for 12 h prior to 12 more consecutive analysis h (n = 6). Respiratory exchange (RER) was measured at post-27 days of treatment. The data are expressed as mean \pm SEM. Black bar represents the dark room cycle with area without representing light room cycle (12 h:12 h).

Fig 6.30

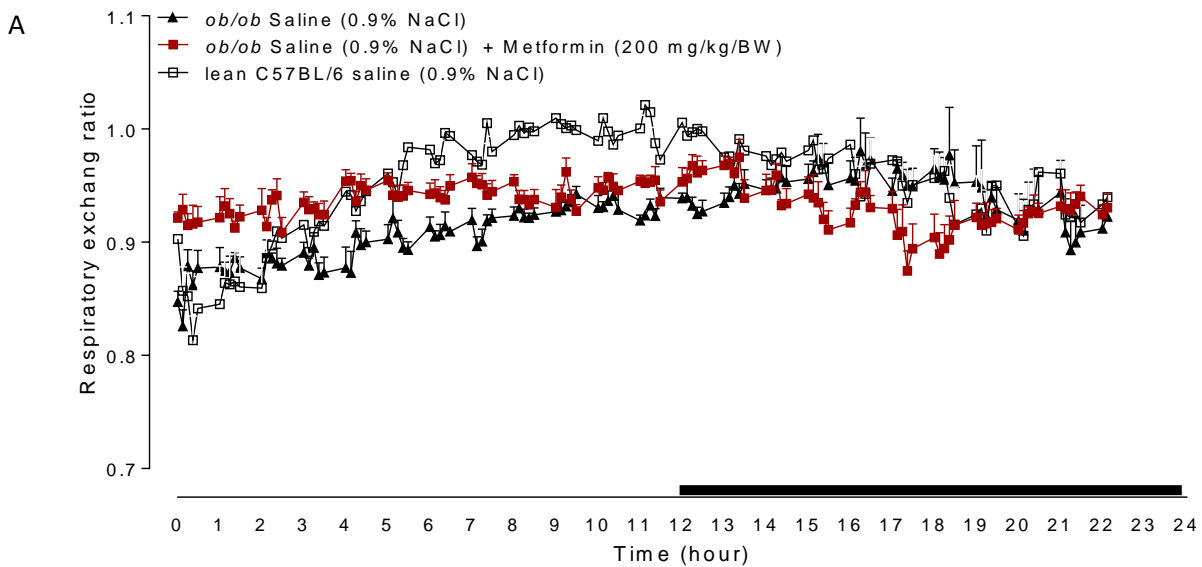


Fig 6.30 (A). Respiratory exchange ratio after twice-daily metformin administration in *ob/ob* mice. The mice were single housed and energy expenditure was normalized for 12 h prior to 12 more consecutive analysis hours ($n = 6$). Respiratory exchange (RER) was measured at post-27 days of treatment. The data are expressed as mean \pm SEM. Black bar represents the dark room cycle with area without representing light room cycle (12 h:12 h).

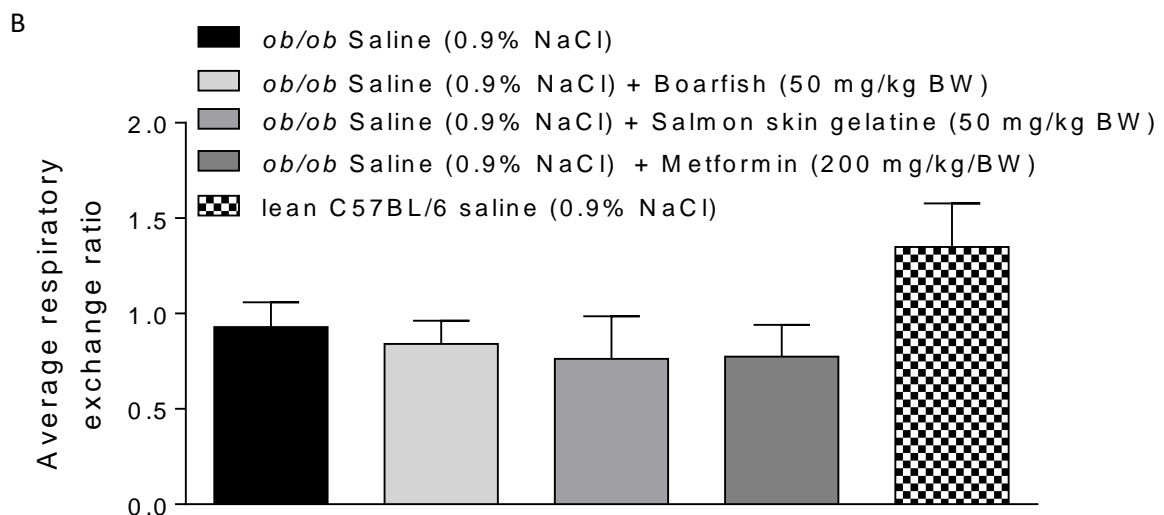


Fig 6.30 (B). Average respiratory exchange ratio post study in *ob/ob* mice. The mice were single housed and energy expenditure was normalized for 12 h prior to 12 more consecutive analysis hours ($n = 6$). Graph represents average VCO_2 (Volume VCO_2) exchanged at post-27 days of treatment. The data are expressed as mean \pm SEM. Black bar represents the dark room cycle with area without representing light room cycle (12 h:12 h).

6.5 Discussion

It is well established that dietary protein components can act as vital regulators of glycaemia through mechanisms that are directly involved in pancreatic insulin secretion or via nutritional stimulation of GLP-1 and GIP via intestinal mechanisms (Thorens, 1995; Shah & Vella, 2015). The post-prandial incretin effect is one of the most important regulators of glucose homeostasis with oral protein hydrolysates acting as moderate inhibitors of DPP-4 of which may well prolong the incretin effect (Tulipano *et al*, 2011). One of the major limitations encountered via the screening of marine protein hydrolysates using *in vitro* and small animal models was the hydrolysis method. Work has shown that sequential hydrolysis of proteins using more than one proteolytic enzyme preparation yields an abundant amount of small molecular weight peptides. This screening approach has resulted in improved bioactivity using cultured cell lines with potent insulin and GLP-1 secretion (Harnedy *et al*, 2018). In small animal models during an oral glucose tolerance test, the co-administration of marine hydrolysates were able to improve post-prandial glucose excursion via the increase of circulating plasma insulin. It is unknown for now, if this effect was via direct pancreatic interaction or via intestinal hormone stimulation or inhibition of endogenous proteolytic enzymes such as DPP-4.

In order to test the oral bioavailability, administration of various marine protein hydrolysates was deployed, the study acknowledges that to exert a systemic effect the peptides must reach their target sites intact (Muheem *et al*, 2016). Previous studies have shown that lab based simulated gastrointestinal digestion (SGID) of blue whiting or boarfish hydrolysates has modest effect on overall bioactivity, and in some cases improved cellular secretion of GLP-1 in cultured cells. However, this approach, not in all cases, provided encouraging results however several enzymatic hydrolysis approaches may hinder bioactivity post-SGID. Hydrolysis using two enzymatic preparations can potentially yield smaller peptides ranging from 2 to 10 amino acids in length, which *in-vitro* investigations demonstrate resistance to further degradation and may therefore improve oral bioavailability. This study investigated boarfish and salmon skin gelatine protein hydrolysates generated using the combination of Alcalase and Flavourzyme food grade enzymes.

Genetically obese diabetic mice (C57BL/6 *ob/ob*) were grouped based upon their non-fasting blood glucose and body weights. No significance difference in either parameter was present between the groups prior to beginning the study. Animals were assigned either physiological saline (0.9% NaCl) or saline containing the treatment that consisted of either boarfish or salmon skin hydrolysate (50 mg/kg bw) or metformin (200 mg/kg).

Non-fasting blood glucose (NF-BG)

Animals were acclimatized using physiological saline from day -3 to -1 and from day 0 treatments were started and administered orally twice daily, once in the AM (e.g. time 09.00 h) and once in the PM (17.00 h). Non-fasting blood glucose (NF-BG) including weight, food intake and plasma were recorded and collected for analysis. During the study, metformin resulted in a significant reduction of non-fasting blood glucose at day 3, however this effect was lost but returned from day 15 onwards until day 27. Both hydrolysate treatment groups showed sporadic improvements in NF-BG levels with boarfish presenting a significant reduction at day 6 initially, and returning from day 15 onwards until completion. Salmon skin gelatine however, resulted in a reduction in NF-BG from day 9 and throughout until day 27 with an overall significant reduction of NF-BG ranging from 20 mmol/L at day 9, reducing to 14 mmol/L by day 27. Similarly, both metformin and boarfish presented reductions in NF-BG concentrations similar to that of salmon skin gelatine, with the saline treated control *ob/ob* group unsurprisingly averaging 31 mmol/L by day 27. Its unclear presently if the peptide components or indeed the further degraded smaller peptides or indeed amino acids generated from the further digestion of the hydrolysates are having the effect seen upon improved NF-BG in *ob/ob* mice.

Non-fasting (NF) plasma insulin concentration

As mentioned, protein components have insulintropic activities, which may be immediate or delayed (Park *et al*, 2015). This may be in part due to the liberated amino acids or indeed the peptide components of protein itself. Our *in vitro* analysis after solid phase and RP-HPLC fractioning, which attempted to remove polar compounds and free amino acids, had yielded potent dose-dependent insulintropic action in cultured cells (refer back to section in thesis). When administered twice daily, both the boarfish and salmon skin gelatine group displayed an increasing trend of NF-blood insulin concentrations. Boarfish presented the only significant increase of plasma insulin at day 12 versus the saline control group, but this effect was lost thereafter. When each group was analysed via area under the curve for NF-insulin through the study, the increasing trend of circulating insulin was significantly elevated within the boarfish hydrolysate treatment group only. This may be explained by an overall improvement of insulin secretion and action from the hydrolysate treatment, In speculating on potential mechanisms in a mouse model that expresses insulin resistance from early stages of life the NF-BG reduction suggests an insulin independent mechanism may be involved. Insulin secretion study in *ob/ob* mice suggest that leptin injections improved glucose-stimulated insulin secretion including a reduction of triglyceride levels in islets, which is further discussed by Khan and colleagues (2001).

Weight changes and food intake during administration of treatments

As previously mentioned, animals were grouped using non-fasting blood glucose concentration and body weight. Throughout the administration of treatments, improvement in NF-BG and modest elevation of NF-insulin concentration, particularly within the boarfish treatment group, were noted. Neither weight reduction or weight gain were not evident in any of the treatment groups. Weight variances between animal groups remained consistent with overall weight gain of each *ob/ob* treatment group averaging between 12 – 14%. Interestingly, an overall reduction in food intake was present in both hydrolysate treatment groups as well as in the metformin group. Unfortunately, this

did not translate into weight loss for any of the animal groups, however studies investigating weight regain in RYGB-ob/ob mice concluded that leptin may prevent weight regain and diabetes recurrence and finally mice continued to gain weight after RYGB surgery was performed (Hao *et al*, 2016). Interestingly, the reduction of food intake and satiety improvements was leptin independent; however, the mechanism by which the hydrolysate treatments reduced food intake is unknown and requires further investigation. Furthermore, protein hydrolysates from many biological sources have reported potent cholecystokinin (CCK) secretion from cultured cells, suggesting CCK release from enteroendocrine cells are nitrogen compound sensing that may elude to the satiety effect within both hydrolysate groups (Foltz *et al*, 2008)

Cumulative food intake and satiety

The effect of protein and increased feeling of fullness and promotion of satiety is well established (Paddon-Jones *et al*, 2008), an effect that varies widely however between protein sources (Du *et al*, 2018). Activation of satiety centres via the promotion of CCK and leptin including reduction of the appetite hormone ghrelin is often considered a viable approach in food studies for combatting weight gain and obesity, with studies suggesting these cannot yet serve as a tool for the satiating effect of foods (De Graaf *et al*, 2004). While limitations have been highlighted, the overall effect of promoting satiety is much more complex with pleiotropic effects of incretin hormones such as GLP-1, which is a potent insulinotropic peptide, that also aids in reducing food intake via promoting a reduction of food intake and improved feeling of fullness. Out of the three main macronutrients including fibre, only protein and fibre have been regarded as potentially viable approaches for obesity prevention (Astrup, 2005; Clark & Slavin, 2013).

During our analysis of food intake in mice, metformin, which is reported to decrease food intake and promote weight loss (Lee & Morley, 1998), had shown a significant reduction in overall food intake from day 9 through 27, with the group averaging a 17% total reduction of food intake

versus the saline control *ob/ob* group. Metformin may act as a DPP-4 inhibitor (Lindsay *et al.* 2005; Green *et al.* 2006; Duffy *et al.* 2007; Cuthbertson 2009) among many other activities. Both boarfish and salmon skin gelatine resulted in a reduction in food intake from day 12, but the effect was lost at day 15 for SSG, but regained from day 18 onward until study completion (day 27). Once a significant reduction of food intake was established with boarfish at day 12, the effect was sustained until completion of the study at day 27. With regards to the overall reduction of total food intake, boarfish resulted in 16% less food intake within the group whereas SSG, while still significantly lower versus the saline control, lowered food intake at 12%. The reduction in overall food intake between each of the treatment groups was not translated into reduction in total bodyweight, however the limitations of using this model for weight reduction studies has been mentioned earlier (Hao *et al.*, 2016). Both sources of protein hydrolysate have not been reported within the published literature for their effects upon food intake until now. However, there are several salmon protein hydrolysate products on the market aimed at reducing body mass index in humans but it is unclear how this assumption is made without human investigation (Hofseth BioCare AS).

Fasting blood glucose and oral glucose tolerance (OGTT) and intraperitoneal glucose tolerance (ip-GTT)

Post-treatments, each group was subsequently fasted for 12 h prior to assessing oral glucose tolerance tests at the end of the 27 day treatment regime. Interestingly, each treatment group averaged a fasting blood glucose concentration of 18 mmol/l (± 2 mmol/L) with the interesting exception of the boarfish hydrolysate treatment group. The boarfish group showed a significant reduction in fasting blood glucose concentration versus the saline control group and returned to almost normoglycaemia with fasting glucose concentrations of 10 mmol/l (± 3 mmol/L). Interestingly, when investigating the acute delayed glucose tolerance effect of boarfish, of all the hydrolysates investigated, only boarfish showed an improvement of glucose tolerance at both 4 h and 8 h suggesting that the effects may be

both acute and longer acting but the associated mechanisms and mode of action are still to be fully elucidated. Studies investigating protein pre-loading may enhance glucose-lowering efficacy of the DPP-4 inhibitor, suggesting the improved fasting glucose levels within the boarfish group may be incretin mediated (Wu *et al*, 2016). Each of the groups after chronic exposure were challenged with 18.8 mmol/kg glucose to test acute glucose tolerance over 120 min. Both hydrolysate treated groups and metformin treated mice had shown significant improvements of glucose concentration at 15 min post-glucose challenge. Improvements were lost from 30 min to 60 min, however both metformin and the salmon skin gelatine treatment groups resulted in significant reduction of blood glucose at both 90 min and 120 min versus the saline control group. The Boarfish hydrolysate treatment group returned to similar levels of both metformin and salmon skin gelatine, however not until 120 min. During analysis of blood glucose area under the curve, again both metformin and salmon skin gelatine hydrolysate group had significantly lower total circulating blood glucose versus the *ob/ob* saline control group. Using a similar approach, administration of the glucose challenge was altered from oral to IP injection using the same concentration of glucose. Initial glucose elevation was similar across all *ob/ob* treatment groups, however by 90 min the boarfish group displayed a significant reduction in circulating blood glucose which was maintained until 120 min, while metformin presented a significant reduction at 120 min only. No improvement in ipGTT was evident within the salmon skin gelatine group with glucose area under the curve showing the same outcome. This approach may suggest that the insulinotropic and delayed effect of glucose tolerance improvements seen with boarfish may be beyond that of the post-prandial incretin effect and potentially via internalised systems, while salmon skin gelatine hydrolysate relies on post-prandial incretin effect via oral intake to achieve biological activity by direct effects within the gut. Future work investigating glucose tolerance may require pre-loading with hydrolysates prior to glucose challenge. A study investigating protein preloading had shown enhanced glucose lowering, improved C-peptide and insulinotropic actions after a high-glycaemic-index breakfast was consumed via augmented GLP-1 action (Jakubowicz *et al*, 2014)

Insulin sensitivity

Classically leptin deficient *ob/ob* mice present insulin resistance, rapid weight gain, obesity and diabetes from early life (Muzzin *et al*, 1996). In these animals, insulin sensitivity was only assessed post-treatments. Each animal was administered via an ip injection a dose of 50 U/kg bodyweight of bovine insulin. Blood glucose concentrations were then measured post-injection (t=0 min) and at 30 min and 60 min. Results indicate that specifically metformin or salmon skin gelatine did not show improvements in insulin sensitivity over the 60 min duration. The boarfish hydrolysate group however did display a modest trend in lowered glucose levels post-insulin injection, however this was non-significant. It is apparent that improvements in insulin sensitivity are derived from changes in body weight, with those who are diabetic at higher BMI ranges requiring larger doses of insulin to get a desired hypoglycaemic effect over those at lower BMI ranges. This approach to insulin sensitivity in *ob/ob* mice was partly expected, due to the relatively large amount of centralised adiposity even after improvements of non-fasting blood glucose and reduction of food intake. To see a marked improvement of insulin sensitivity in this animal model, a significant reduction in body weight or particularly, adipose reduction, is required (Meade, 1978). This was not evident within the present study and with the caveat for a potential limitation of using this mouse model for weight change and insulin sensitivity analysis (Hao *et al*, 2016).

Terminal glycated haemoglobin A1c (HbA_{1c})

The relevance of HbA_{1c} is now known to be an invaluable diagnostic tool for monitoring long-term glycaemic control (Weykamp, 2013). The measurement of glycated haemoglobin A1c is now the gold standard for establishing long term glycaemic control and commonly deployed alongside glucose tolerance prior to a T2D diagnosis. Typical ranges of HbA_{1c} vary between 4% to 6.5%, with ranges between 5.7% and 6.4% categorised as pre-diabetes or over 6.5% as diabetes (WHO, 2011). Post-treatment analysis of each of the hydrolysate treatment groups including the metformin group resulted in a significant reduction in HbA_{1c} concentrations. The saline control groups HbA_{1c} was 8%

(±1%) whereas better improvement of HbA_{1c}, resulted from boarfish returning 5.6% versus 6.1% for that of the salmon skin gelatine treated group. The metformin treatment group averages a HbA_{1c} score of 6.2%. These HbA_{1c} scores for the metformin and SSG are still elevated, however in comparison to the control mice HbA_{1c} was dramatically improved over the *ob/ob* saline treated control group. Interestingly, the boarfish HbA_{1c} score of 5.6% would classify the group as having well-controlled long-term blood glucose and falling within non-diabetic range. Interestingly, high protein diets have beneficial effects on weight loss, which can be translated to improvement in HbA_{1c} concentrations; however, the effect is seen mainly with long-term intake of protein, at significantly higher amounts than administered within the present study (Dong *et al*, 2013). It is perhaps not surprising that each of the *ob/ob* treatment groups resulted in improved HbA_{1c} compared to the *ob/ob* saline control group due to the reduction of food intake including the improved non-fasting blood glucose profiles.

Terminal plasma GLP-1 concentrations

Glucagon-like-peptide 1 (GLP-1), a hormone secreted from the intestinal enteroendocrine L-cells following nutrient stimulation is a potent insulin secretagogue, as well as having notable effects upon satiety, delaying gastric emptying and β -cell proliferation. The hormone is unfortunately rapidly degraded via the serine peptidase, dipeptidyl peptidase-IV (DPP-4). Several pharmacological approaches for treating T2D have been mentioned, however in short, the promotion of GLP-1 using nutritional factors could be a highly beneficial approach to better management of glucose homeostasis in individuals with T2D (Bodnaruc *et al*, 2016). Following the chronic treatment of mice over a 30-day period, a one off fasting sample of total GLP-1 was analysed. Interestingly, the boarfish hydrolysate treated group had a 46% increase in circulating plasma insulin concentration compared to the saline treated *ob/ob* control group. There was no change in total GLP-1 present within the SSG hydrolysate treatment group, however rather surprisingly the metformin group showed a 25% reduction versus the saline treated *ob/ob* control group. Across all groups there was elevated total

GLP-1 levels versus the lean saline control group, however this is not surprising due to the nature of post-prandial GLP-1 secretion, where *ob/ob* groups averaged 25 – 40% additional food intake per day. Within the total GLP-1 analysis it is not possible to distinguish between the bioactive isoforms of GLP-1(7-36)amide of GLP-1(7-37) versus the assumed inactive truncated GLP-1(9-37) degradation product. The elevated GLP-1 levels however might partially explain the food intake reduction that was evident within the boarfish group. Glucagon-like-peptide 1 has been associated with pancreatic β -cell CCK secretion including elevated levels of GLP-1 involved in a reduction of abnormal hepatic lipid retention (Linnemann *et al*, 2015). Rather surprisingly, metformin-induced GLP-1 secretion is reported within the literature (Cuthbertson *et al*. 2009) with dual combination therapies taking advantage of this by combining metformin with DPP-4 inhibitors within a single pill. Again, without establishing which GLP-1 isoforms are predominantly present within the blood circulation system makes the consequences of a reduction of GLP-1 noted with metformin treatment group difficult to predict.

Circulating plasma lipids

Screening of circulating plasma lipids is now commonplace in diabetes, obesity and inflammatory related diseases, with hyperlipidaemia frequently present among poorly controlled T2D sufferers (Daniel, 2011). Blood plasma lipids were analysed for cholesterol, high density lipoprotein (HDL), low density lipoproteins (LDL) and triglycerides. Each of the *ob/ob* treatment groups had significantly elevated cholesterol over the lean saline control animals; however, but when compared to the *ob/ob* saline treated group, both SSG and metformin both resulted in a significant reduction of total plasma cholesterol. A potentially misleading outcome noted, after HDL analysis, showing an almost 1.5- fold increase in total circulating HDL versus the lean control group across all *ob/ob* treatment groups. It is important to note that studies looking specifically at HDL turnover are advised to investigate using a more appropriate animal model as leptin deficient mice as such, present defective HDL particle uptake with hindered recycling and degradation of HDL resulting in abnormally plasma HDL concentrations (Silver *et al*, 2000). To our knowledge, this is the only aspect of the *ob/ob* lipid profile that raised

concerns. The boarfish, SSG including metformin treatment groups showed significantly reduced plasma triglycerides versus the saline treated *ob/ob* controls. The plasma triglyceride concentration from each of the treatment groups matched that of the lean saline control group indicating marked improvement in diabetic status. Furthermore, HbA_{1c} could be correlated with triglyceride status and a predictor of diabetic control, since treatment groups had shown a significant improvement of both HbA_{1c} and circulating triglycerides post treatment (Naqvi *et al*, 2017). A study investigating salmon protein hydrolysates generated via adjusted hydrolysis approaches had shown very different effects of oral administration of the hydrolysate source on hepatic enzymes. The peptides generated were from different regions of the salmon body with molecular weights averaging 200 – 1200da or above. That study concluded that improvements in plasma lipid concentrations was due to interaction with various hydrolysate peptide fractions and their effect on enzyme desaturases which in turn resulted in altered lipid parameters including weight loss (Vik *et al*, 2015). Furthermore, a study investigating the mechanism by which protein hydrolysates affect lipid metabolism concluded that lipid-independent atherosclerotic activity was present in ApoE-deficient mice with the ability to attenuate risk factors relating to atherosclerotic risk that were independent to changes in plasma lipids or fatty acids (Parolini *et al*, 2014).

Isolated pancreatic islet β -cell functionality tests performed post-study

Alterations to both islet cell morphology including density and secretory capability is adversely affected in the diabetic state, including severe pathognomonic changes (Gepts & lecompte, 1981). Within our own analysis of islet functionality post-treatment of isolated pancreatic islets, we reported comparable insulin secretion when compared to effects of cellular secretagogues. In particular, during insulin secretion investigations, there no increase of insulin secretion over that of lean mice islets, with cells responding to potassium chloride (KCl), and GLP-1 in a similar manner. However when analysed for insulin content post-acute analysis, the *ob/ob* mice indeed presented elevated internalised insulin content versus that of the lean control animals with high glucose (16.7 mM) alone,

or in combination with KCl or GLP-1 showing a 3-fold rise in insulin content versus the lean control mice, but no significant differences between the *ob/ob* treated saline controls or treatment groups, respectively. On subsequent analysis of pancreatic hormone content investigated using 20 mg of pancreas and acid ethanol hormone extraction, protein analysis returned significantly lower pancreatic protein content within the boarfish and SSG treatment groups. Using the same approach for determining total pancreatic insulin content yielded similar insulin content between each of the *ob/ob* animal models with lean control animals on average showing 30% less insulin content. Without identifying total pancreatic hormone content, in particular, glucagon and pancreatic polypeptide (PP) it is unclear at present, how the decreased total protein content represented within hydrolysate groups with overall similar insulin content to other groups was of benefit to the general diabetes status. Comparing this however, the total hormone content versus total insulin would represent significantly higher pancreatic insulin content within these *ob/ob* mice.

Cellular morphology analysed using immunohistochemistry

Changes within the pancreatic islets were analysed using fluorescent microscopy. Excised pancreata were stained with primary mouse antibodies for insulin and glucagon, and there was particular interest in α to β -cell ratio. Impaired insulin secretion is a consequence of functional and survival defects at the cellular level, hallmarked by abnormal basal insulin secretion and loss of first-phase insulin secretion (Del Prato & Marchetti, 2004). Studies suggest that both pancreatic defects including a lower number of beta cells and alteration to alpha cell mass may contribute to diabetic progression (Henquin & Rahier, 2011). As the diabetic state progresses, studies have reported increased cellular differentiation and cellular survival via α to β -cell trans-differentiation (Lui & Habener, 2009). Correcting both beta- and alpha cell dysfunction becomes an attractive therapeutic approach. Firstly, the average cell area was assessed between the *ob/ob* mice groups. The *ob/ob* mouse model has increased islet volume however this does not translate into increased islet functionality. Islets within

lean animals are significantly smaller, and yet possess the same secretory capacity. On analysis, both boarfish and metformin treated groups had significantly smaller islet volume versus the saline *ob/ob* control and SSG groups. Analysis of insulin cell area within total islet area returned various results, again with boarfish and metformin groups with lower insulin cell area, whereas SSG with significantly elevated insulin cell area. Analysis of the average glucagon cell islet area showed that boarfish and SSG had lower average glucagon cell area, whereas no change was noted with metformin compared to the saline *ob/ob* control. Finally, the alpha cell to beta cell ratio of both boarfish and SSG were both significantly lower, resulting in less alpha cells within the islet. The *ob/ob* control group alpha cells consisted of 6% of the total islet mass, whereas boarfish and SSG averaged 4% and 4.2%. These small changes in α -cell to β -cell ratios may suggest a protective mechanism involved via protecting β -cells from damage causing cellular transdifferentiating to α -cells, however more mechanistic investigations are warranted.

Anthropometric and body composition analysis using DEXA

Animals were analysed for intergroup changes of body composition including fat mass, lean body mass and bone mineral density (BMD) and bone mineral content (BMC). DEXA analysis of total body BMD had shown no changes within each of the *ob/ob* mouse groups, with elevated BMD within the lean control animals. Interestingly however, total BMC was significantly improved within both the boarfish and SSG hydrolysate treatment groups and BMC was comparable to the lean saline control group; however, a significant reduction of BMC was noted in the metformin group compared to the *ob/ob* saline treated control group. Studies investigating metformin using ovariectomy rodent models reported no differences in bone architecture compared to a saline control over an 8-week treatment period (Jeyabalan *et al*, 2013). Within the literature, BMD is reportedly affected within the diabetic state (Ma *et al*, 2012). The increase of BMC within the hydrolysate groups might possibly be explained via dietary calcium binding properties of small molecular weight peptides. Indeed the calcium chelating properties of some amino acids and small peptides for improved calcium bioavailability has

been reported (Sun *et al*, 2016; Zhao *et al*, 2014). Increased calcium binding affinity has been noted with peptides such as Gly-Leu and Gly-Gly which are abundant in marine collagen (Tang & Skibsted, 2016). Analysis of lean mass revealed that no changes between treatment and non-treatment groups including no change recorded within the lean control mice. Furthermore, as expected with genetically obese animal models, fat mass was over 4-fold elevated between *ob/ob* animals and the lean control group. The fat mass between the *ob/ob* treatment and *ob/ob* control group displayed no differences indicating that the reduction of food intake, including the improvement of non-fasting blood glucose parameters had no effect on adipocyte turnover and utilization as a fuel source with similar results found with total body fat content. The body composition of the *ob/ob* animals still consisted primarily of fat mass, with each of the *ob/ob* group's post-study maintaining body weight based upon 50% contributed solely from fat mass alone. This was reflected when identifying how lean body mass was affected with all *ob/ob* animal models with a similar return of 50% lean mass, whereas due to the relatively low fat mass of the lean control animals, their total lean mass percentage was 80%.

Oxygen exchange ratios analysed using CLAMS

Pre-loading of particular macronutrients for improved oxygen exchange in athletes is commonly investigated (Woodward & Bird, 1991). Within this study, the elevated body mass of the *ob/ob* mice resulted in significantly lower VO_2 score, VCO_2 score and average respiratory exchange ratio (RER) compared to the lean control group. Group variances between *ob/ob* treatment groups were not significantly different in comparison to the *ob/ob* saline control group. No improvements in respiratory exchange ratios were noted, which may reflect the relative lack of weight loss noted within each of the *ob/ob* treatment groups.

Conclusion

The results presented herein report a high throughput hydrolysis screening approach using both *in vitro* to *in vivo* aiming to unravel the multifaceted effects and importantly the oral efficacy of protein hydrolysate intake from two different marine sources and assess their anti-diabetic effects. Further mechanistic analysis using acute and long-term biomarker approach is acknowledged; however, the research has highlighted glucoregulatory activity of marine protein hydrolysates, which will be summarised. This study was not designed to directly compare food derived biomolecules against a known and highly regarded oral hypoglycaemic agent, however the incorporation of metformin was for control purposes only from a tried and tested anti-diabetic pharmaceutical. With this noted, the study has highlighted several key aspects of small molecular weight peptides from marine sources and their ability to promote satiety and lowering of food intake, improvement of non-fasting and fasting blood glucose including better circulating plasma lipid profiles and HbA1c. Furthermore, outcomes and results relating to bone mineral content, pancreatic cell morphology and inflammation are aspects that the researcher will aim to unravel within a future study. Finally, the most bioactive hydrolysate from the study is boarfish, with the work generated on its lasting effects over the shorter lived salmon skin gelatine (SSG) hydrolysate has indicated that boarfish protein hydrolysate is a target that would benefit from further investigations in a human trial.

Chapter 7

Investigating long-term twice-daily oral intake of Algal protein hydrolysates derived from *Palmaria palmata* (Dulse) in multiple low dose-streptozotocin diabetic induced mice

7.1 Introduction

The last several decades have seen the most dramatic changes in dietary intake patterns. Access to food, including foodstuffs from different continents has become easier to obtain. Unfortunately, free movement of food across global networks has not improved population health with controversially the opposite is often the case (Marlow *et al.*, 2009; Popkin & Kenan, 2016). Nevertheless, the opportunity to alter dietary intake based upon dietary needs is now available with a vast array of food varieties. Climate change and the rise in global population and has unfortunately placed tremendous strain on the food chain, with access to high quality protein being a priority in the need to meet ever growing global demands (Luber & Prudent. 2009; Hall *et al.*, 2017). The ability to exploit all potential food sources and nutrients requires substantial economic investment and support (Proietti *et al.*, 2015).

Underutilized nitrogen sources such as marine waste byproducts are classically incorporated within low-cost animal feed or fertilizers (Sranacharoenpong *et al.*, 2015). Similarly, demand for protein and its peptide components has seen an exponential growth within the cosmetics industry (Secchi, 2008). Furthermore, sustainable protein sources are abundant, such as animal and plant-based being readily available albeit, these sources are not without their economic costs (Rosi *et al.*, 2017). The financial cost of protein food choices between biological sources are noted with aim to reduce the environmental footprint worldwide. This includes dietary protein source changes to prevent the prevalence of non-communicable diseases such as obesity and diabetes (Musaiger & Al-Hazzaa, 2012). Swelling demand for high quality animal-based proteins will place increased pressure on global resources which contributes to greenhouse gas emissions including requiring more water and land (Sabaté *et al.*, 2014).

The issue surrounding food security is not only restricted to terrestrial protein sources as evidenced from recent changes to EU fishery policies relating to marine and oceanic sustainability which has seen larger volumes of commercially underutilized pelagic species landed (Pauly & Zeller, 2016; Manach *et al.*, 2013). Industry, researchers and government have adapted rapidly and prioritised the task of maximising the utilization of protein containing marine sources (Gajanan *et al.*, 2016; Slizyte *et al.*, 2016; Harnedy *et al.*, 2018). Closer to land, coastal sustainability of marine alga sources has similarly been emphasised (Rebours *et al.*, 2014; Monagail *et al.*, 2017). The worldwide seaweed industry produce many products from seaweed components either for direct or indirect human use. Macroalgae and microalgae are the most abundant coastal ecosystems and play vital role as habitats for invertebrates, fish, mammals and birds (Bishop *et al.*, 2017). The nutritional importance of edible seaweeds should not be underestimated due to their favourable fibre, lipid, carbohydrate, mineral, vitamin and protein content (Norziah & Ching, 2000; Cian *et al.*, 2015). Edible macroalgae was once a staple food within the western diet, and remains as one within far eastern countries including China and Japan (Teas *et al.*, 2013). The resurgence of European seaweeds tailored towards disease states has recently shifted with advice to avoid certain sources of seaweed due to potential mineral toxicity (Clarke *et al.*, 2003; Zava & Zava, 2011).

The protein content between seaweeds vary dramatically ranging from 5% to 40% of dry weight. The protein content of seaweeds depends particularly on species, environmental conditions and the season in which harvested (Cerna, 2011, 1999; Khairy & Shafay, 2013). Algal protein is of particular interest due to the favourable amino acid composition containing all essential amino acids including glycine, alanine, proline and glutamic and aspartic acids (Bleakley & Hayes, 2017). Red seaweeds are particularly interesting as these have the highest

reported algal protein content, with some reaching 40% of dry weight (Fleurence & Dumay, 2018). Proteins with physiological relevance may be an attractive approach using seaweeds as a source of ingredients with high nutritional quality.

As mentioned previously, the readily available access to diverse foodstuffs has its limitations in terms of health implications. Population rise of obesity and diabetes has reached epidemic proportions placing major strain on health care systems (Kim & Basu, 2016). Type 2 diabetes (T2D) cases are expected to rise from 340 million presently, to 640 million by 2040 (Marín-Peñalver *et al.*, 2016). Currently type 2 diabetes is the most common metabolic and endocrine disorder and now considered the global epidemic of the century (Golden *et al.*, 2009; Kharroubi & Darwish, 2015). Body systems maintain glycaemia within narrow parameters following food intake, however individuals diagnosed with T2D, hyperglycaemic, pancreatic dysfunction, altered insulin secretion, insulin resistance and insensitivity are unfortunately very common making up >5-10% of the global population (Færch *et al.*, 2015). The need for modern reinvention of dietary approaches to prevent or manage obesity and related metabolic diseases such as T2D is very necessary. The metabolic benefit of protein in helping improve glycaemic regulation is well established, however the source of protein and quantity needed to enhance its therapeutic potential is not well established. It is however well recognised,, that under most conditions, protein provides an increased satiety response which lowers subsequent food intake than the isoenergetic ingestion of fat or carbohydrates. Despite this some studies report that the satiating effects of certain proteins are not evident. The digestion rate of protein and subsequent rate at which liberated amino acids or small peptides are absorbed and presented in the blood circulation will affect bioavailability. Thus protein digestion products may have influences upon improved insulin secretion, post-prandial incretin secretion and satiety. It is now becoming clear, that the amino acid

composition of the protein does not always correlate with improvements relating to these pathways.

Evidence now argues liberated peptide fragments of protein, known as protein hydrolysates including small molecular weight peptides (SMWPs), can promote activation of satiety centres via cholecystokinin and leptin production, both potent satiety-promoting hormones (Add reference here e.g. Caron *et al.*, 2016). The benefits of ingesting protein hydrolysates are not limited to satiety, with studies displaying potent post-prandial insulinotropic hormone secretion including glucagon-like-peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which are associated with beneficial effects upon satiety, insulin secretion and delayed gastric emptying. The post-prandial effect of GLP-1 and GIP is unfortunately short-lived, due to rapid degradation of both hormones by the serine protease, dipeptidyl peptide 4 (DPP-4) (Deacon, 2011).

Protein hydrolysates and their constituent peptides have reported DPP-4 inhibitory activity in both *in vitro* and *in vivo* experimental models (Harnedy *et al.*, 2017). Recently bioactive peptide fragments identified from *Palmaria palmata* have displayed DPP-4 inhibitory activity *in vitro*. Proteins from casein and whey fractions of milk have similarly been reported to contain DPP-4 inhibitory activity *in vitro*. Proteins from marine collagen and fish contain high proline residues, may also present DPP-4 inhibitory activity (Xia *et al.*, 2017). Work carried out investigating the anti-diabetic activity of fish proteins have shown some promising data relating to bioactivity responses within culture cells and small rodent models. Both salmon and blue whiting protein hydrolysates reported potent dose-dependent insulinotropic activity including GLP-1 secretion *in vitro* (Harnedy *et al.*, 2017; Harnedy *et al.*, 2018). Furthermore, the oral availability of protein hydrolysates and bioactive peptides reaching

target cells intact is problematic. Despite this, improvement in post-prandial glycaemic and enhanced insulinotropic responses were found following oral administration of blue whiting protein hydrolysates after an OGTT glucose tolerance test (Harnedy *et al*, 2018) There are several amino acids of which are known to promote insulin and GLP-1 secretion, particularly alanine and glutamine, which are abundant in marine proteins. It is expected that small bioactive peptides may be closely involved in stimulating the insulinotropic responses.

The screening of protein derived from algal species is not without concerns. In particular, the sustainability and reproducibility of the algal source including preferential harvesting seasons to maximise protein yield is acknowledged. With this noted, characterising the therapeutic effect of algal proteins targeting the treatment of T2D is nevertheless a worthwhile endeavour. This study aimed to explore the satiety and glycaemic effect of protein extracted from *Palmaria palmata* protein hydrolysate abundant in SMWPs within a Streptozotocin induced diabetic mouse model.

Background & Aims

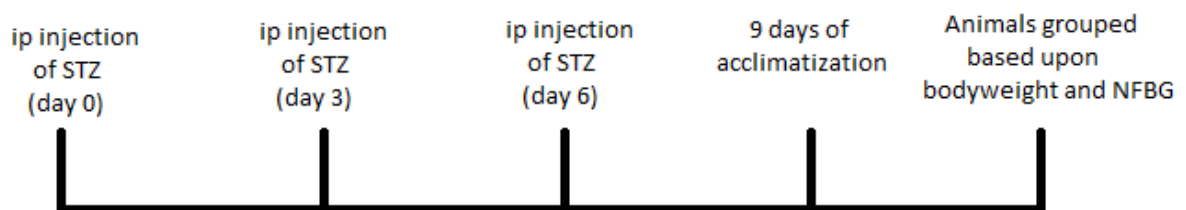
The intake of seaweed has been linked to improved health in countries that habitually incorporate it within their diet including lower incidence of certain cancers including improved iodine and fibre status, however the effect of seaweed proteins on health is still within its infancy. Seaweeds vary in protein content, with red seaweed, *Palmaria palmata*, local to Irish and northern European shores containing the highest protein content, however varying from 10% to 30% (w/v) dependent on seasonality. Using a similar approach as the previous chapter, the aim of this work is to investigate the effect of long-term twice-daily oral intake of seaweed protein hydrolysates in diabetes-induced mice with focus on food intake, non-fasting glucose levels and anthropometric and bone parameters. We hypothesize that

similar to the previous work carried out, a reduction of food intake will be evident, promoting improvements in non-fast blood glucose, fasting blood glucose levels suggesting metabolic improvements in animals lacking insulin production.

7.2 Materials and Methods

Establishing diabetes and grouping

Using an animal model described in 2.3.3, 10 week old (n=32) *HsdOla:TO* mice were administered several low doses of the β -cell toxin Streptozotocin (40 mg/kg bodyweight x 3 on day 1, 4 and 7 pre-grouping). Prior to treatment commencing animals were firstly acclimatized for 3 days (study day -3 to day -1) with twice daily oral saline (0.9% NaCl) gavage (9 am + 5 pm) to mimic treatments of which started on study day 0.



Glucose tolerance tests (intraperitoneal and oral)

As described in section 2.2.3 and 2.3.4 blood samples were collected from a minor tail vein bleed in fasted (8 h) mice. Blood glucose was measured using a handheld glucometer (Bayer Contour, Leverkusen, Germany) prior (t=0) to administering a glucose challenge (18.8 mmol/kg/ bodyweight). Once administered (either i.p or oral), blood glucose was further analysed at 15, 30, 60, 90 and 120 min.

Tissue excision

On completion of all experiments mice were fasted (4 h) and sacrificed. Mice were placed unconscious via oral inhalation of a general anaesthetic (Isoflurane) and euthanized via cervical dislocation. Tissue excision protocol explained in detail in section 2.4.6.

Pancreatic hormone content

Thawed tissue was rinsed in cold PBS before being weighed and transferred to a bijoux containing 2 ml of ice cold acid ethanol (1.5% (v/v) HCl (12.5 M), 75% (v/v) ethanol, 23.5% (v/v) H₂O). Described in detail in section 2.4.7 and 2.4.8

Assessment of pancreatic insulin and glucagon content

Pancreatic homogenates were diluted to a range of concentrations (1:100, 1:200, 1:500 and 1:1000) using stock RIA buffer prepared in section 2.2.2 and tested for insulin content using the RIA described in 2.2.3.

Total pancreatic protein content

Insulin RIA and glucagon ELISA were used to determine total pancreatic hormone content. In order to quantify detected hormone percentage it was compared to total pancreatic protein concentration from tissue homogenate described in section 2.4.7.

Assessment of terminal plasma lipid profile

Terminal plasma sample lipid profile was determine using an I-Lab 650 clinical chemistry system (Instrumentation Laboratory, Warrington, UK). The analysis consisted of assessing total triglycerides, total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) concentration. Reagents for triglyceride analysis were obtained from Instrumentation Laboratory (Warrington, UK) and reagent for LDL cholesterol were obtained from Randox Healthcare (Randox, Co, Antrim, N. Ireland).

Statistical analysis

Data was expressed as mean \pm SEM with values compared using one-way analysis of variance (ANOVA) unless otherwise stated. Data between groups were considered statistically significant if $p \leq 0.05$. Data was plotted and analysed using GraphPad Prism 5.

7.3 Results

Fig 7.1 – Animal grouping post-streptozotocin (STZ) injection acclimatization

Groups of mice were given an i.p. injection of STZ (40 mg/kg/bw) once every 3 days for 9 days and given a further 9 days for acclimatization. Once all STZ-injected animals had shown significant elevation of non-fasting blood glucose (cut-off 10 mM) they were subsequently group based upon non-fasting blood glucose (NFBG) and body weight. All STZ-treated mice by day 20 had displayed significantly elevated ($p < 0.001$) NFBG compared to their pre-STZ injected state (Fig 7.1).

Fig 7.2 – Effects of twice daily treatment on non-fasting blood glucose parameters

Animals were assigned various treatments; including physiological saline only within STZ-injected mouse groups or a non-STZ control group. Other groups consisted of STZ-injected mice given twice daily treatments of either *P. palmata* protein hydrolysate (PPPH, 50 mg/kg/bw) or metformin (200 mg/kg/bw) dissolved in a physiological saline carrier. Every third day NFBG was measured using a handheld glucometer. A significant improvement in NFBG was evident within the metformin group from day 9 to completion of the study at day 18 ($p < 0.01$ – $p < 0.001$) and similar improvements displayed within the PPPH group from day 9 to 18 also ($p < 0.01$ – $p < 0.001$) versus the STZ-saline control group, respectively (Fig. 7.2A). Total NFBG was analysed via area under the curve, with both treatment groups resulting in a significant reduction of NFBG over the study duration in the PPPH ($p < 0.001$) and metformin ($p < 0.001$) versus the STZ saline control group. The non-STZ control group had consistently lower NFBG than all STZ groups (Fig. 7.2).

Fig 7.3 - Effects of twice daily treatment on non-fasting plasma insulin parameters

Non-fasting plasma insulin concentrations were similarly analysed using RIA every third day. Mice all displayed significantly lower circulating plasma insulin concentrations versus the non-STZ control group (Fig7.3). Furthermore, the PPPH treatment group displayed elevated circulating insulin concentrations from day 6 through day 18 of the study (day 6 $p < 0.01$ – day 18 $p < 0.001$) except on day 12, whereas the metformin group only showed elevated plasma insulin concentrations on day 6

($p < 0.05$) and day 18 ($p < 0.001$), both versus the STZ saline treated control group. Total plasma insulin analysed via area above the curve (AAC) similarly resulted in significantly higher plasma insulin levels within the PPPH ($p < 0.001$) and metformin ($p < 0.001$) versus the STZ saline treated control group (Fig. 7.3B).

Fig 7.4 - Effects of twice daily treatment on energy intake

Food intake was assessed over the 18 day treatment period and converted into energy intake (1 g of food = 12.99 kJ of energy). A reduction of food intake was within the *Palmaria palmata* protein hydrolysate (PPH) group at day 15 (25% reduction, $p < 0.05$) and day 18 (24% reduction, $p < 0.01$) versus the STZ saline treated control group (Fig. 7.4). No significant change in energy intake was noted within the metformin group versus the STZ control group. The normal control mice which were not given STZ injections consumed less food than STZ saline treated mice on day 15 (30% reduction, $p < 0.05$) and day 18 (33% reduction, $p < 0.05$) (Fig. 7.4).

Fig 7.5 A - Effects of twice daily treatments on long-term glucose control analysed using glycated haemoglobin (HbA_{1c})

Following the completion of the intervention period terminal HbA_{1c} was analysed using the HbA_{1c} analyser (A1cNow) kit. The STZ saline treated control group showed HbA_{1c} levels of 10.5%, which was significantly reduced in the PPPH treatment group (7.2%; $p < 0.01$) and metformin group (6.8%; $p < 0.001$) (Fig. 7.5A). The control mice which were not injected with STZ had the lowest HbA_{1c} values (4.0%; $p < 0.001$) versus the STZ saline treated group (Fig. 7.5A).

7.5 B - Effects of twice daily treatment on fasting glucose concentration

Blood glucose concentrations were significantly improved following an 8 h fasting period when compared to STZ saline treated mice. Blood glucose was measured from tail blood via a handheld Bayer Countour glucometer. Both of the STZ treatment groups resulted in improved fasting glucose, with PPPH producing an average of 12.6 mmol/L ($p < 0.01$) and metformin group showing 17.1 mmol/L

($p < 0.05$) versus the severely hyperglycaemic STZ saline treated control group ((28.7 mmol/L). Fasting blood glucose for the control mice not injected with STZ were within the normoglycaemic physiological glucose range of 5.4 mmol/L (Fig. 7.5B).

Fig 7.6 – Effect of twice daily treatments on oral glucose tolerance responses

Mice were fasted for 8 h and blood glucose levels analysed prior to glucose challenge ($t = 0$ min). Glucose was administered orally (18.8 mM) and subsequently blood glucose concentrations measured at regular intervals up until 120 min (Fig. 7.6). Improvements in glucose tolerance were noted at each time point from 0 min through 120 min within both the PPPH groups ($p < 0.01$ – $p < 0.001$) and within the metformin treated group ($p < 0.05$ – $p < 0.01$) compared to the STZ saline treated control group. Total blood glucose concentration was analysed using area under the curve (AUC), again with both treatment groups resulting in significantly lower blood glucose concentrations (PPPH $p < 0.05$; metformin $p < 0.01$) versus the STZ saline treated control group (Fig. 7.6). The best oral glucose tolerance responses was shown by the mice which had not been treated with STZ injections ($p < 0.001$, Fig. 7.6).

7.7 A – Measurement of the percentage bodyweight changes following interventions in STZ mice

The weight of each animal was measured every 3 days. Changes in body weight percentages were analysed via pre- and post-treatment weight on day 0 and day 18. A small reduction in weight was noted within the STZ saline control group (1.5%), however a significant reduction of 6.8% was noted within the STZ PPPH group ($p < 0.01$) and 4.9% within the STZ metformin group ($p < 0.05$) (Fig 7.7A). In contrast the mice which did not received STZ injections nshowed a 10% increase ($p < 0.001$) in bodyweight gain over the same 18 day study (Fig. 7.7A).

7.7 B – Assessment of total plasma GLP-1 concentrations following interventions in STZ mice

Following the completion of the 18 day intervention period plasma total GLP-1 was analysed using an ELISA. Compared to the STZ saline treated control group, the PPPH resulted in a significant reduction

of circulating GLP-1 ($p < 0.01$) which was similar to the control mice which were not injected with STZ ($p < 0.001$) Fig 7.7B. There was no significant difference in the total GLP-1 plasma concentrations between the STZ saline treated control and the STZ metformin treatment group. (Fig 7.7 B)

Fig 7.8 A – Analysis of post-treatment circulating plasma triglyceride concentrations following interventions in STZ treated mice

There was no difference in triglyceride concentrations between the STZ saline treated control, the STZ metformin treatment group nor the mice which were not injected with STZ (Fig. 7.8A) . However in contrast, the STZ PPPH treatment group displayed a significant reduction in plasma triglyceride concentration ($p < 0.05$) versus the STZ saline treated control group (Fig. 7.8A).

Fig 7.8 B – Analysis of post-treatment circulating plasma cholesterol concentration

There was no change in cholesterol concentrations between the STZ saline treated controls and the STZ metformin treatment group. However, the STZ PPPH treatment group displayed a significant reduction in plasma total cholesterol concentration ($p < 0.01$) versus STZ saline treated control mice. The mice which were not injected with STZ also showed a reduction in total cholesterol, but this was less marked than the PPPH mice ($p < 0.05$) (Fig 7.8B).

Fig 7.9 – Analysis of total pancreatic glucagon and protein content

Excised pancreas from each treatment group was analysed for pancreatic glucagon content via ELISA. Hormones were extracted in acid-ethanol using 30 mg of tissue and homogenised (See section 2.8). On analysis, there was no significant difference in glucagon content between any of the STZ treatment groups including the control mice not injected with STZ (Fig7.9A). Excised pancreas from each treatment group was analysed for pancreatic protein content using the Bradford colorimetric assay.

Peptides were extracted using acid-ethanol using 30 mg of tissue followed by homogenization. Results showed, there was a significant reduction in total pancreatic protein content within the STZ PPPH group ($p < 0.001$) the STZ metformin group ($p < 0.01$) and furthermore within the non-STZ saline control group ($p < 0.05$) versus the STZ saline treated control mice (Fig. 7.9B).

Furthermore, when the pancreatic glucagon content was expressed as a percentage of the total protein content extracted, the STZ injected PPPH and metformin groups, as well as the mice which were not injected with STZ showed a trend towards increased glucagon:total protein ratio but this failed to reach significance (Fig. 7.10).

Fig 7.11 A– Effects of chronic treatments of STZ mice on bone mineral content (BMC) and bone mineral density (BMD) using DEXA

Bone mineral content was elevated within the STZ PPPH treatment group ($p < 0.01$) and STZ metformin treatment group ($p < 0.01$) compared to the STZ saline treated control group (Fig. 7.11A). The BMC of the PPPH treatment group was also elevated versus the group of control mice not given STZ injections ($p < 0.05$). This increase was also supported by an increase in the femur BMC ($P < 0.05$). No differences were found between the STZ saline treated and the normal healthy mice who did not receive the STZ injection (Fig. 7.11A). No changes in bone mineral density were found between any of the control or treatment groups (Fig. 7.11B).

Fig 7.13 Effect of treatment of STZ mice on lean body mass (g) and body fat mass using DEXA

No significant changes were reported within lean body mass analysis between any of the the STZ groups. However, the mice who did not receive STZ injections showed a significant rise in lean mass versus the STZ saline treated control mice ($p < 0.01$; Fig 7.13A).

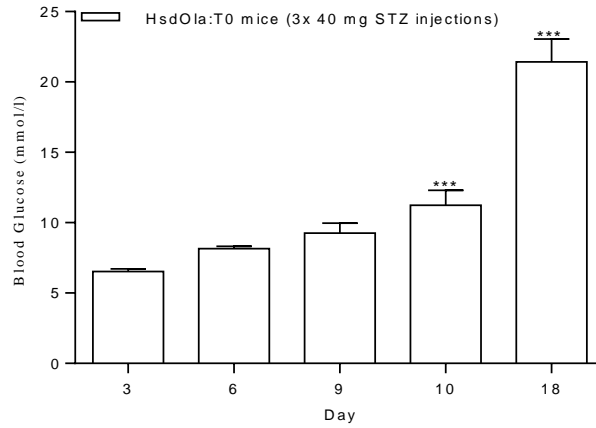
No significant changes were found in fat mass analysis between the STZ groups. However, the mice who did not receive STZ injections showed a significant increase in body fat mass versus the STZ saline treated control mice ($p < 0.01$; Fig 7.13B).

When the % body fat was calculated this was higher for the STZ PPPH treatment group ($p < 0.05$), the STZ metformin treatment group ($p < 0.001$) and the group of control mice not given STZ injections ($p < 0.001$), compared to the STZ saline treated mice (Fig. 7.14A). When the lean mass and fat mass values were added together there was no difference in values for STZ PPPH nor STZ metformin treated mice versus the STZ saline treated controls (Fig 7.14B). However, the control mice not given STZ injections showed a greater body mass ($p < 0.001$), compared to the STZ saline treated mice (Fig. 7.14B).

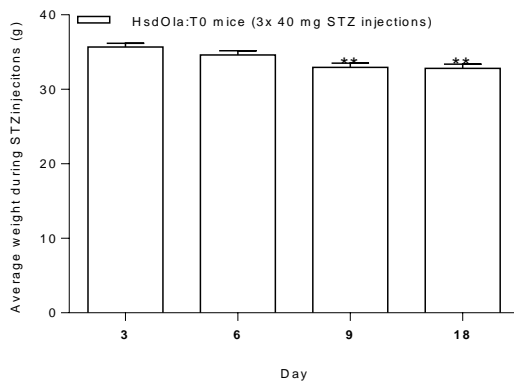
Fig 7.1.

7.3 Results

A



B



C

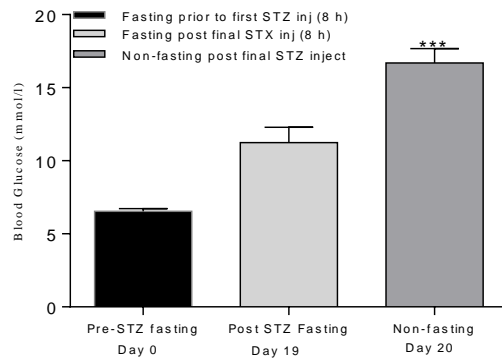


Fig 7.1. Animals were given 3 doses of STZ (40 mg/kg/bw) over a 9 ay period and monitored for **(A)** non-fasting blood glucose and **(B)** body weight including observation of **(C)** pre- and post STZ injection on NF-BG. Both blood glucose and weight were significantly elevated versus lean control group. Results are Mean±SEM for 7-8 observations per group. **p>0.01, ***p<0.001 change versus starting timepoint. Animals were subsequently groups post analysis of day 18 NF-BG.

Fig 7.2

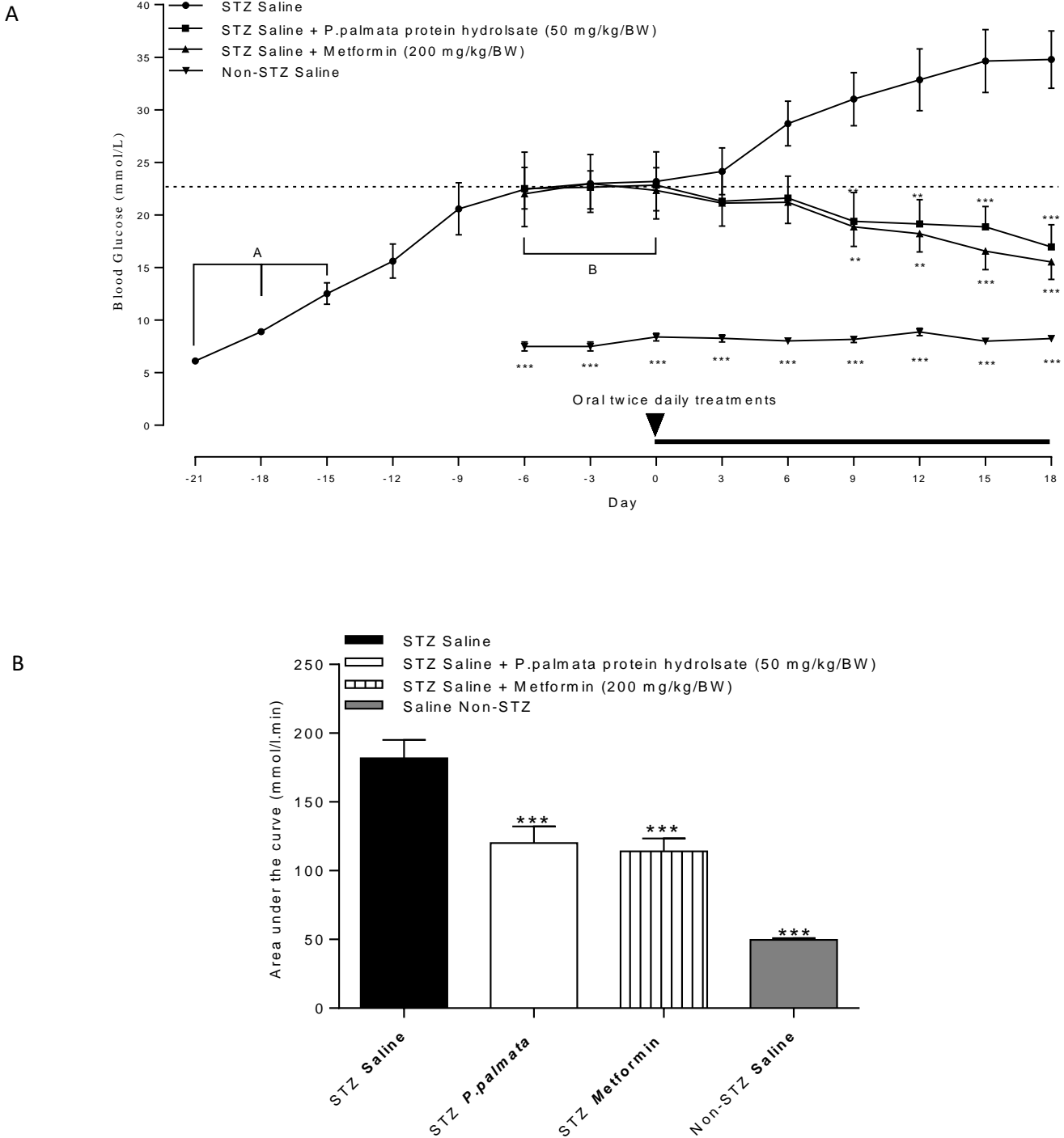
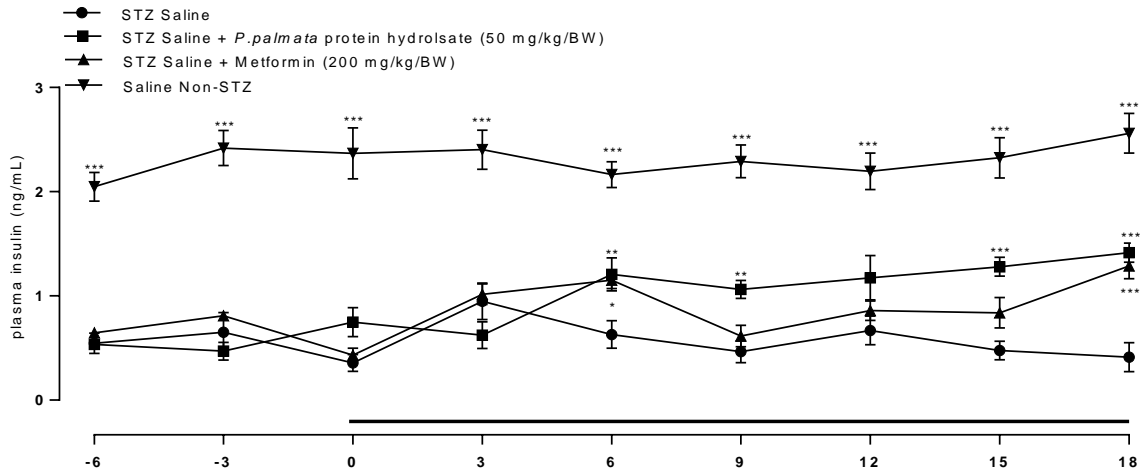


Fig 7.2 Effects of twice-daily administration of palmaria palmata hydrollysate and metformin on non-fasting blood glucose in diabetes induced *STZ* mice (**A**). Parameters were measured 6 days prior with treatment commencing at day 0 to day 18. Twice daily treatment with palmaria palmata hydrollysates (50 mg/kg bw) and metformin (200 mg/kg bw) or saline vehicle (0.9% (w/v) NaCl) in both a *STZ* and non-*STZ* control groups. Values represent mean \pm SEM for 7-8 mice. ** $p < 0.01$, *** $p < 0.001$ vs *STZ* saline control. (**B**) represents Mean \pm SEM blood glucose area under the curve (AUC). *** $p < 0.001$ versus *STZ* saline control, respectively.

Fig 7.3

A



B

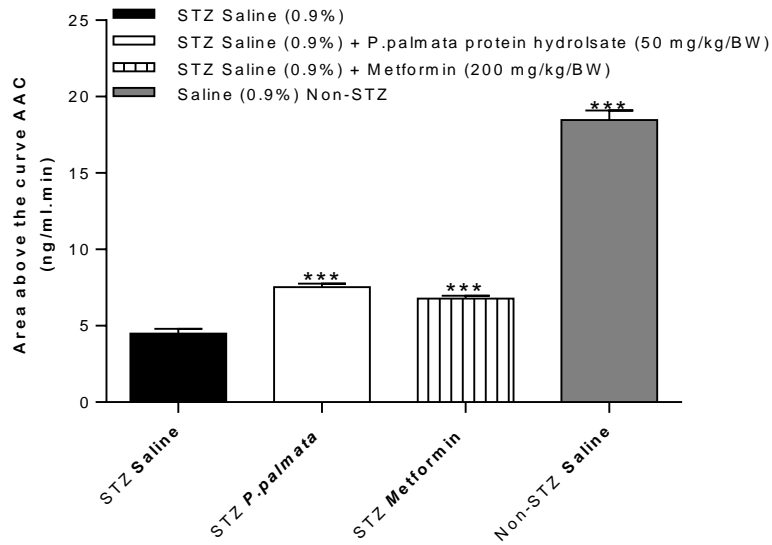


Fig 7.3. Effects of twice-daily administration of *Palmaria palmata* hydrolysate and metformin on non-fasting plasma insulin concentration in diabetes induced STZ mice (A). Parameters were measured 6 days prior to and during treatments commencing at day 0 to day 18. Twice daily treatment with *Palmaria palmata* hydrolysates (50 mg/kg bw) and metformin (200 mg/kg bw) or saline vehicle (0.9% (w/v) NaCl) in both a STZ and non-STZ control groups. Values represent mean \pm SEM for 8 mice. *** $p < 0.001$ vs STZ saline, (B) represents Mean \pm SEM plasma insulin area under the curve (AUC). *** $p < 0.001$, versus STZ saline control.

Fig 7.4

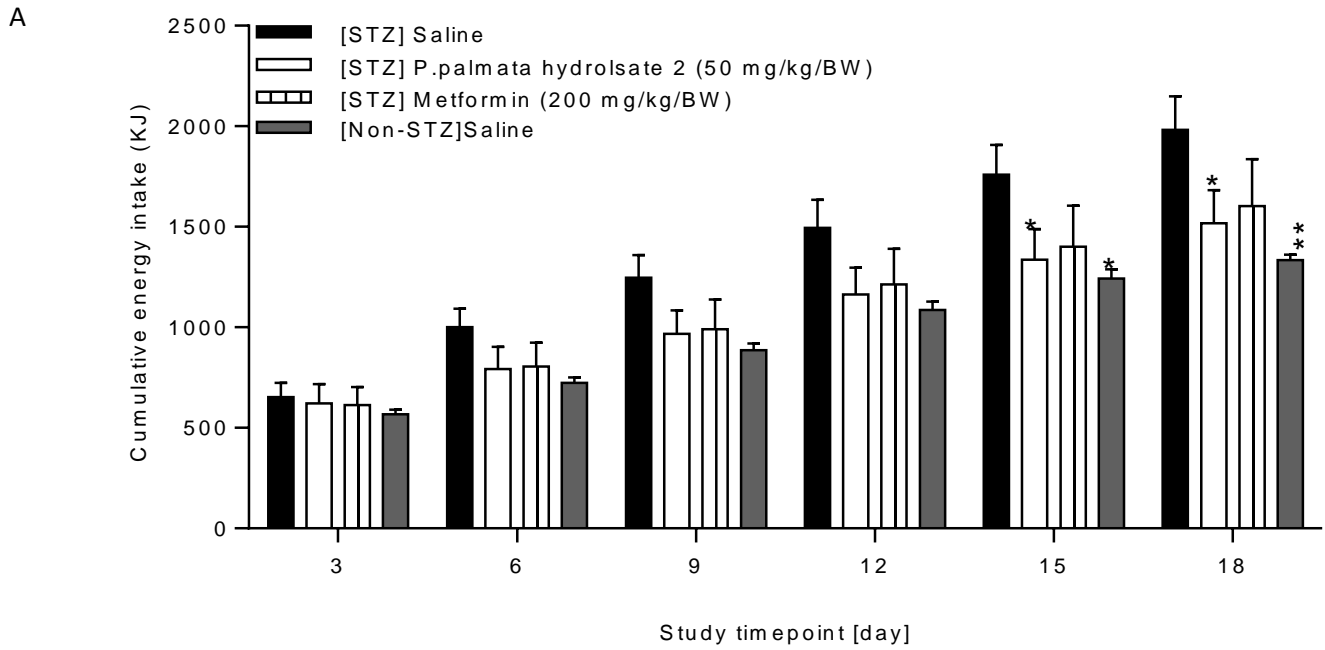


Fig 7.4 Effects of twice-daily administration of marine hydrolysates on energy intake in STZ treated mice. Food was measured every 3 days until day 18 following daily treatment with marine hydrolysates (50 mg/kg bw), metformin (200 mg/kg bw) or saline vehicle (0.9% (w/v) NaCl). Values represent mean \pm SEM for 7-8 mice. * $p < 0.05$ and ** $p < 0.01$ versus the STZ saline treated control group.

Fig 7.5

A

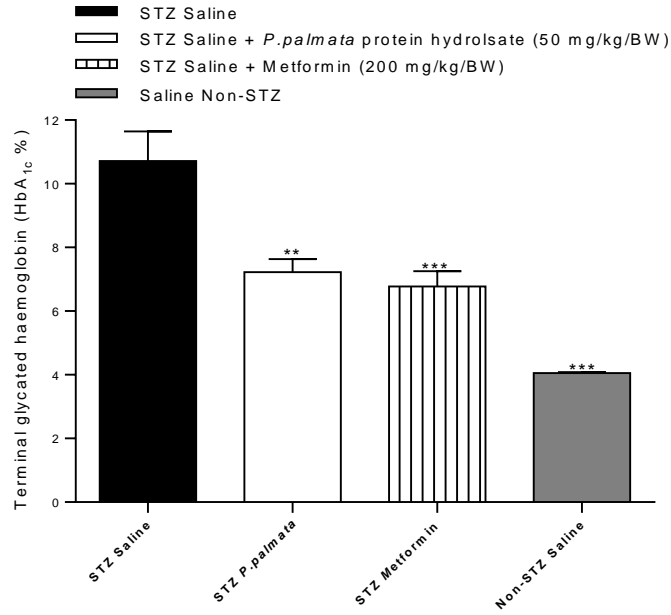


Fig 7.5 A. Effects of twice-daily administration of PPPH and metformin on terminal glycosylated haemoglobin (HbA_{1c}). Values represent mean \pm SEM for 8 mice. *p<0.05, **p<0.01, ***p<0.001 versus STZ saline treated control group.

B

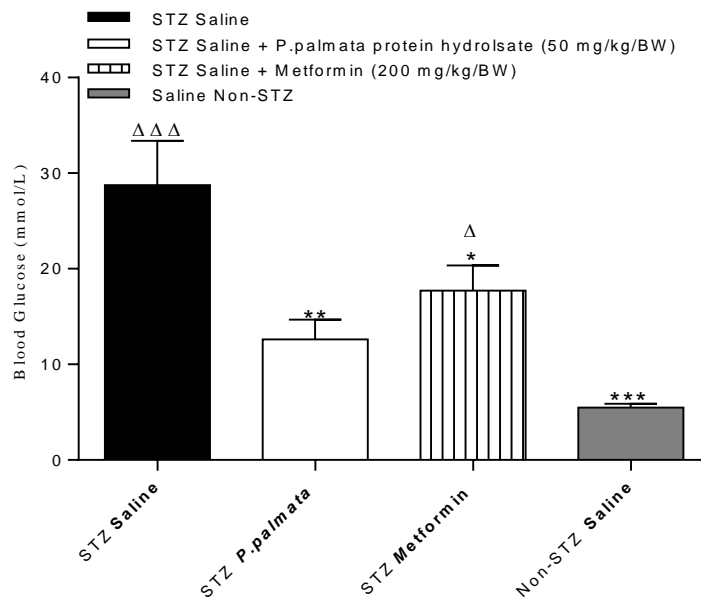
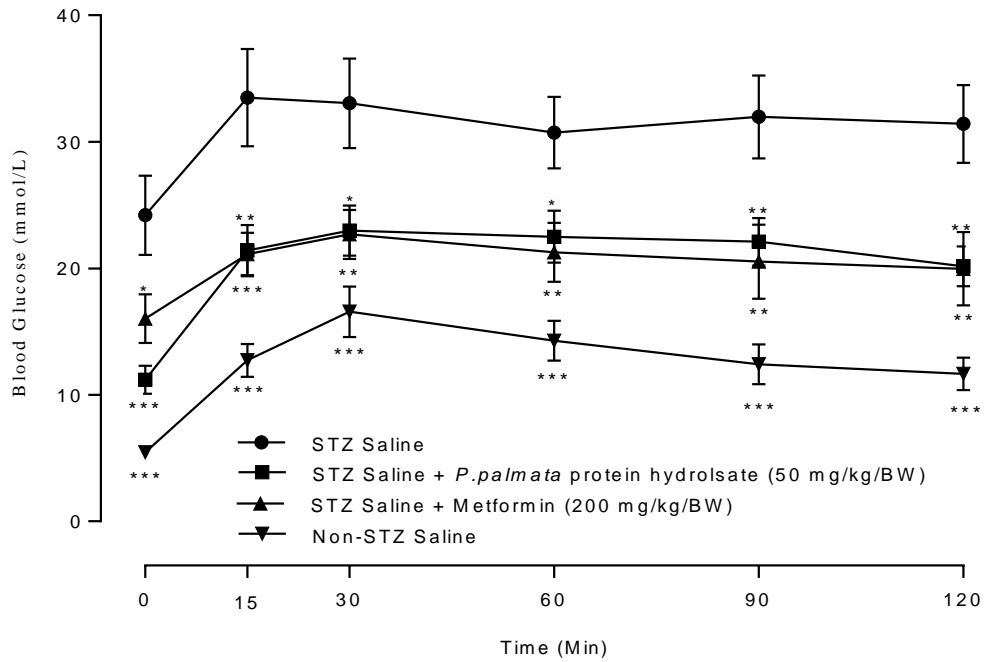


Fig 7.5 B. Effects of twice-daily administration of PPPH and metformin on fasting blood glucose. Mice were fasted for 12 h prior to measurement of glucose. Values represent mean \pm SEM for 7-8 mice. *p<0.05 versus STZ saline control group. Δ p<0.05, $\Delta\Delta\Delta$ p<0.001 versus the non-STZ saline treated control group.

Fig 7.6

A



B

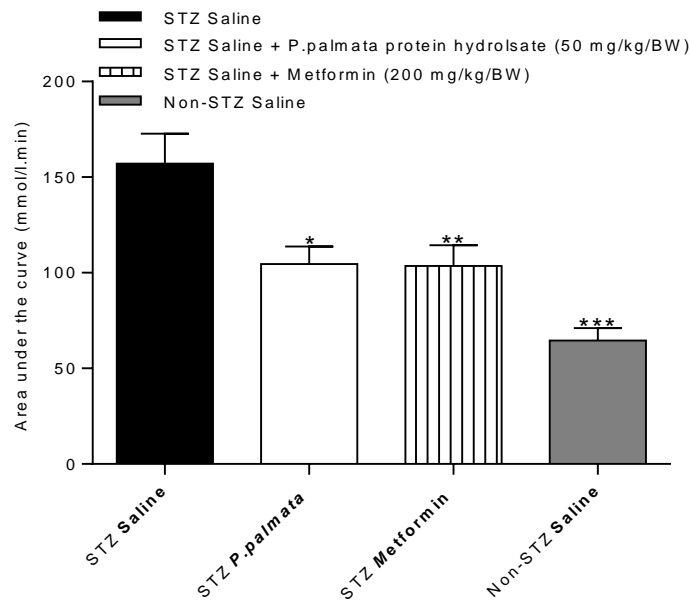


Fig 7.6. Effects of chronic treatment with twice-daily administration of PPPH or metformin on **(A)** terminal oral glucose tolerance and **(B)** integrated OGTT AUC values. Parameters were measured prior to (t=0) and 15, 30, 60, 90 and 120 min after administration of glucose alone (18 mmol/l kg body weight; oral gavage). Glucose AUC values for 0-120 min post-injection. Values are expressed as mean \pm S.E.M. for 8 mice. * $p < 0.05$, and ** $p < 0.01$, *** $p < 0.001$ compared to STZ saline treated controls.

Fig 7.7

A

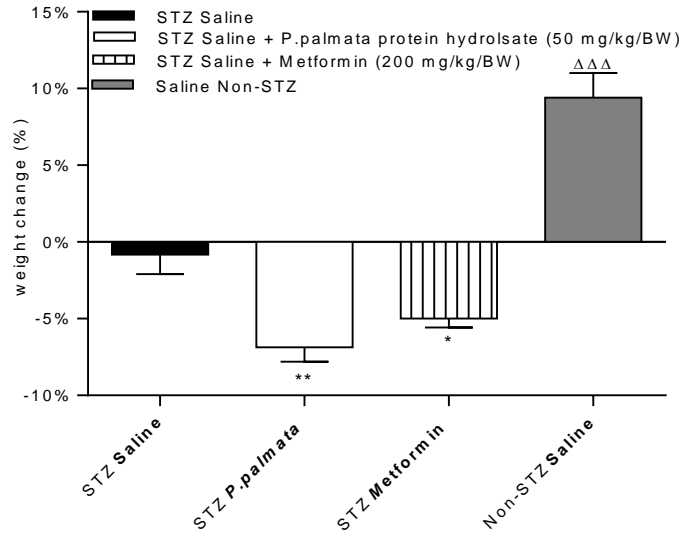


Fig 7.7. Effects of twice-daily administration of PPPH or metformin on percentage weight change (**A**) in STZ mice. Weight was measured 3 days prior to and 18 days during twice daily treatment with marine hydrolysates (50 mg/kg bw), metformin (200 mg/kg bw) or saline vehicle (0.9% (w/v) NaCl). Values represent mean \pm SEM for 7-8 mice. * $p < 0.05$, ** $p < 0.01$ versus STZ saline treated control group.

B

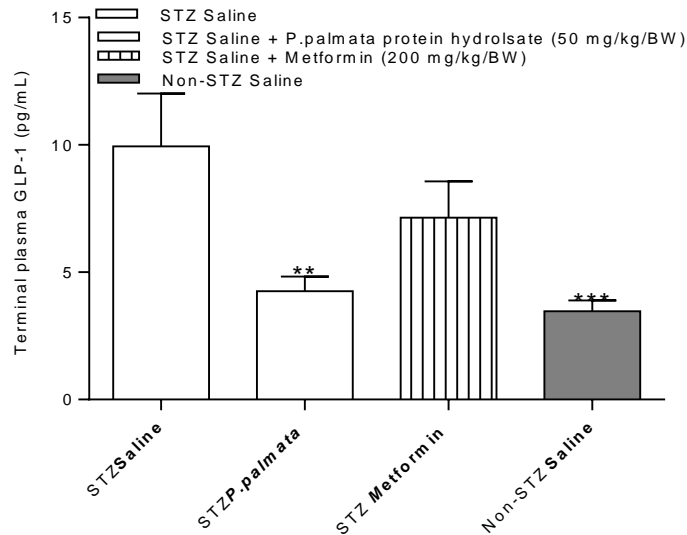


Fig 7.7 B. Effects of twice-daily administration of PPPH or metformin on terminal plasma total GLP-1 concentration. Values represent mean \pm SEM for 8 mice. ** $p < 0.01$, *** $p < 0.001$ versus STZ saline treated control group.

Fig 7.8

A

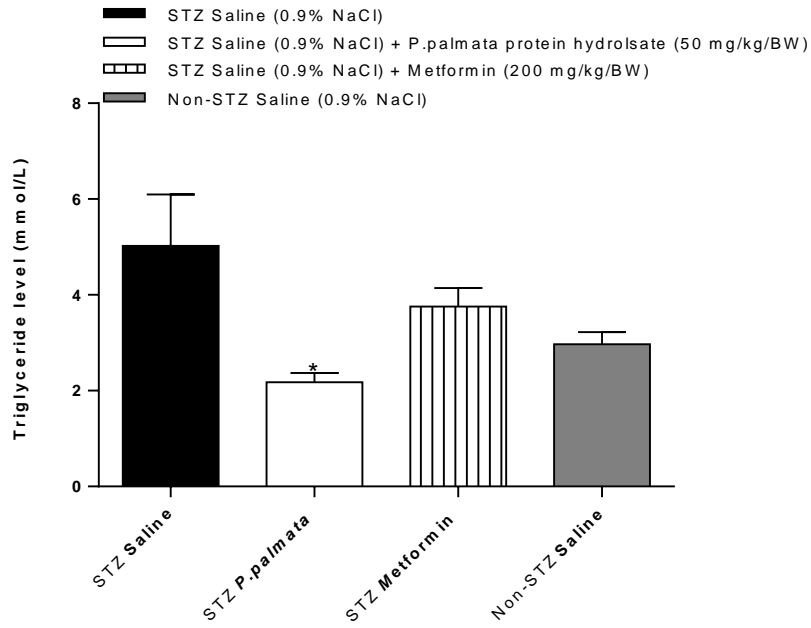


Fig 7.8 A. Effects of twice-daily administration of PPPH or metformin on terminal plasma triglyceride concentrations. Values represent mean \pm SEM for 8 mice. * $p < 0.05$ versus STZ saline treated control group.

B

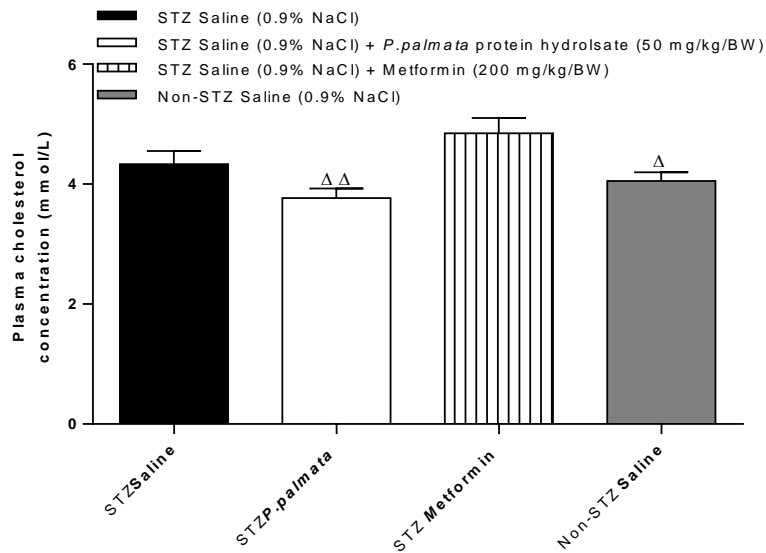


Fig 7.8 B. Effects of twice-daily administration of PPPH or metformin on terminal plasma total cholesterol concentration. Values represent mean \pm SEM for 7-8 mice. $\Delta p < 0.05$, $\Delta\Delta p < 0.01$, versus STZ metformin group.

Fig 7.9

A

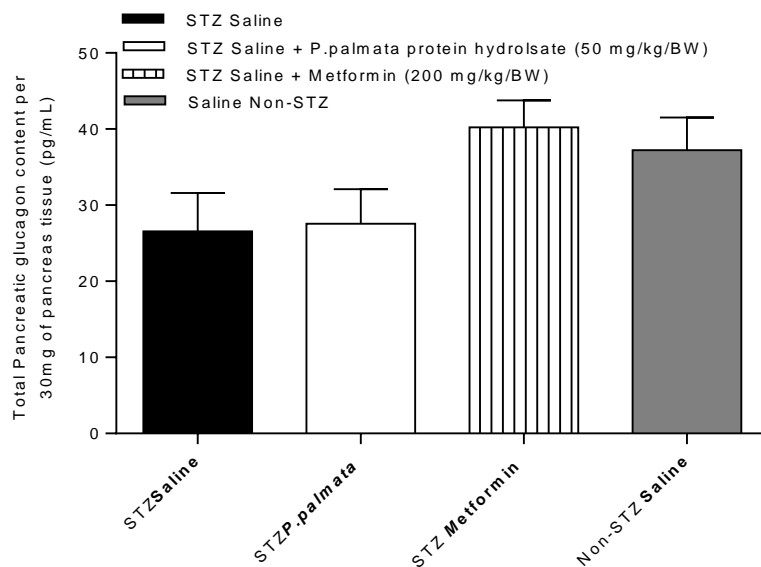


Fig 7.9 A. Effects of twice-daily administration of PPPH or metformin on terminal pancreatic glucagon concentrations. Values represent mean \pm SEM for 8 mice.

B

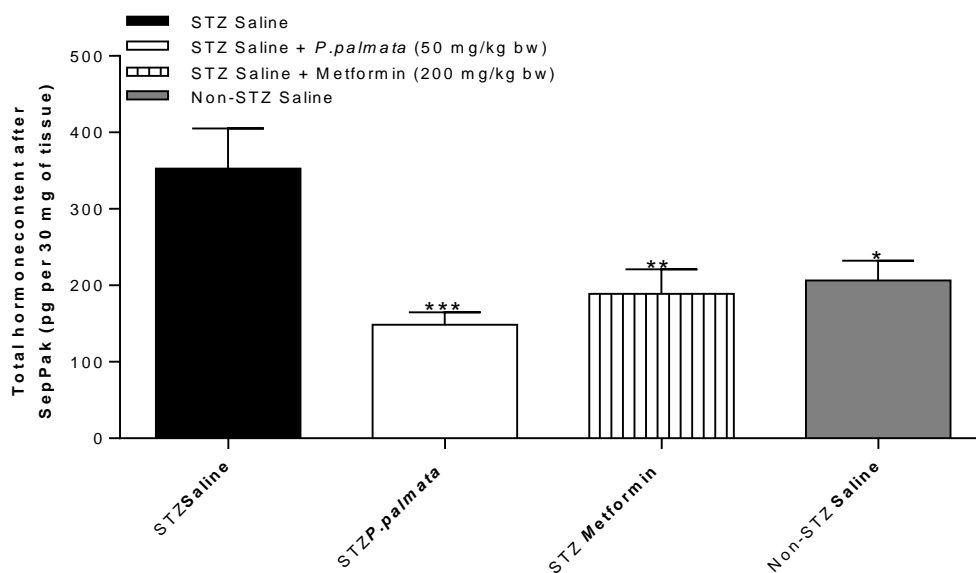


Fig 7.9 B. Effects of twice-daily administration of PPPH or metformin on terminal pancreatic hormone content analysed via Bradford assay. Values represent mean \pm SEM for 8 mice. *p<0.05, **p<0.01, ***p<0.001 versus STZ saline treated control group.

Fig 7.10

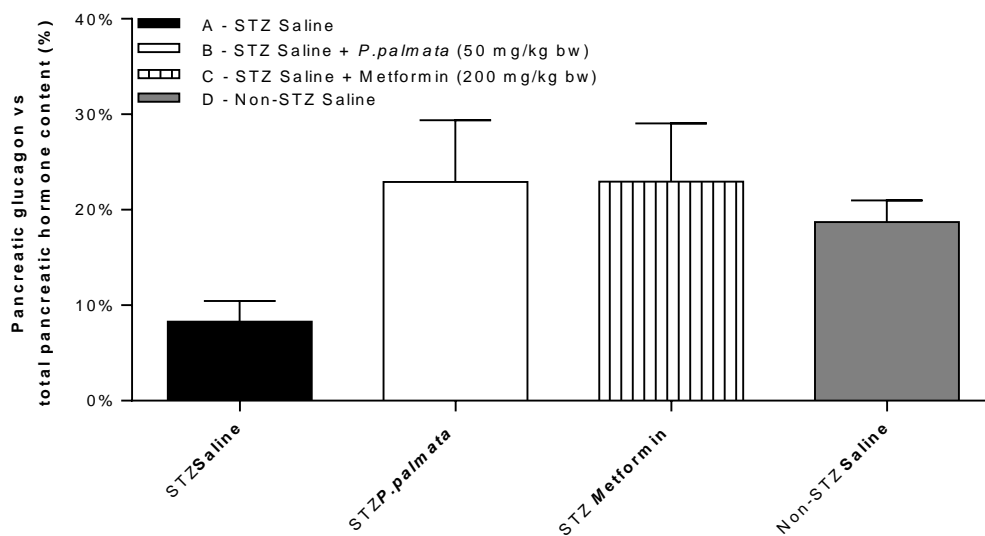


Fig 7.10. Effects of twice-daily administration of *palmaria palmata* on terminal pancreatic glucagon vs hormone content analysed via Bradford assay and glucagon ELISA. Values represent mean \pm SEM for 7-8 mice.

Fig 7.11

A

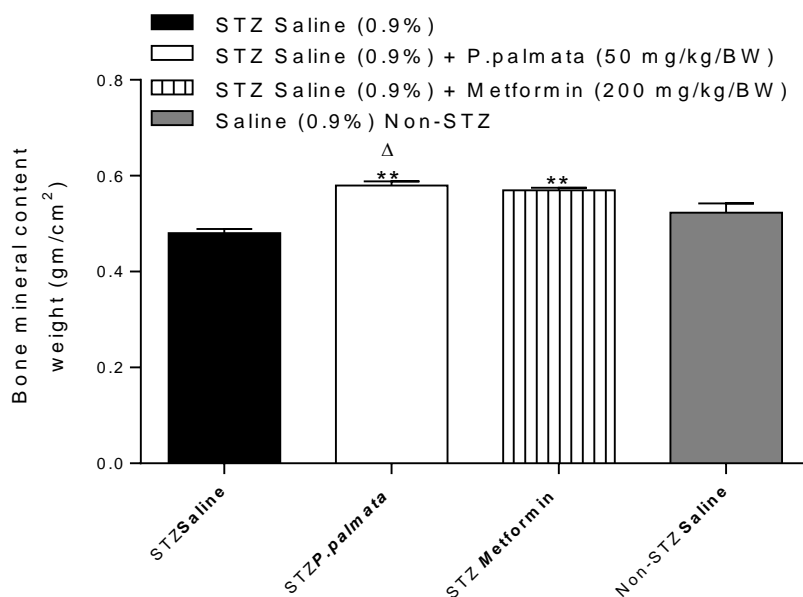


Fig 7.11 A. Effects of twice-daily administration of PPPH or metformin on bone analysis (BMC) assessed by PIXImus DEXA. Graph represents total body bone mineral content (BMC g/cm²). Values represent mean ± SEM for 8 mice. **p<0.01, versus STZ saline treated control group and Δp<0.05 versus non-STZ saline control, respectively.

B

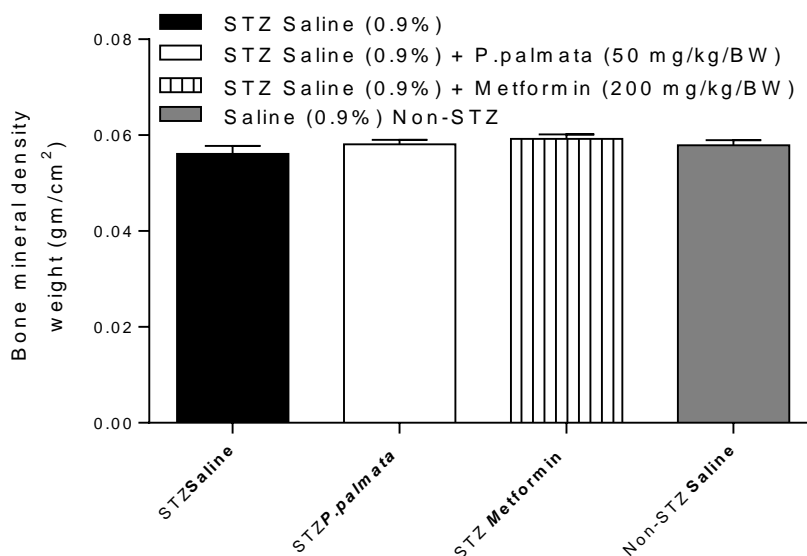


Fig 7.11 B. Effects of twice-daily administration of *palmaria palmata* hydrolysate on bone mineral density (BMD) assessed by PIXImus DEXA. Graph represents total body bone mineral density (BMD g/cm²) Values represent mean ± SEM for 8 mice.

Fig 7.12

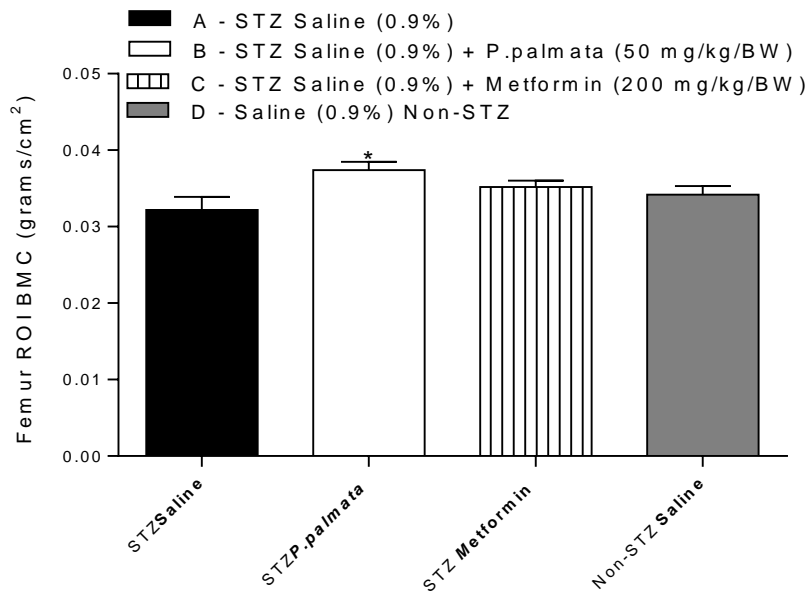


Fig 7.16. Effects of twice-daily administration of PPPH or metformin on anthropometric analysis (Femur ROI BMC) assessed via PIXImus DEXA. Values represent mean \pm SEM for 7-8 mice. * $p < 0.05$, versus STZ saline treated control group.

Fig 7.13

A

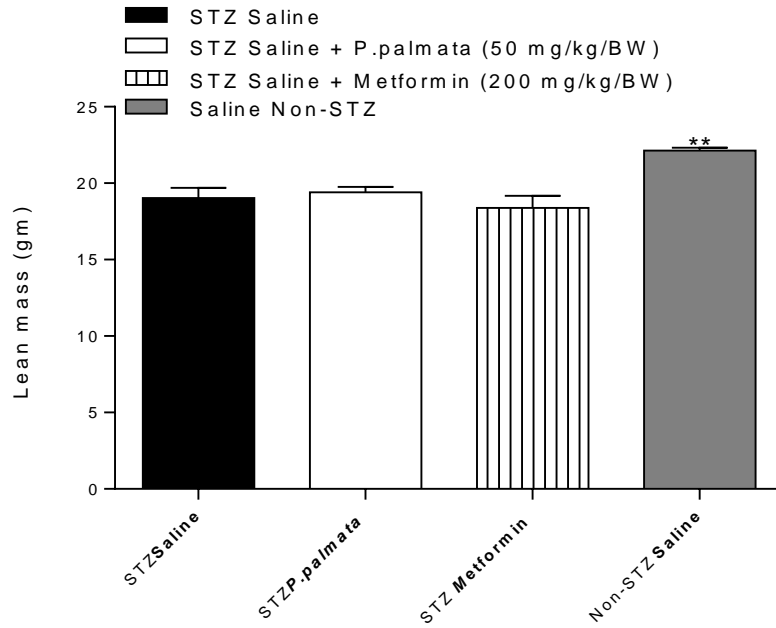


Fig 7.13 A. Effects of twice-daily administration of PPPH or metformin on anthropometric analysis as assessed via PIXImus DEXA. Graph represents total body lean mass (g). Values represent mean \pm SEM for 7-8 mice. * $p < 0.05$, versus STZ saline treated control group.

B

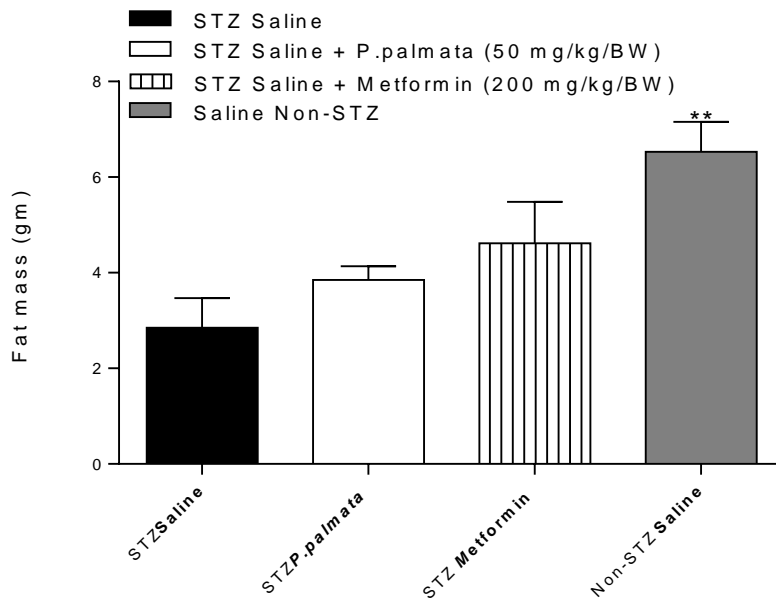


Fig 7.13 B. Effects of twice-daily administration of marine hydrolysates on anthropometric analysis assessed via PIXImus DEXA. Graph represents total body fat mass (grams) Values represent mean \pm SEM for 7-8 mice. ** $p < 0.01$, versus STZ saline control group, respectively

Fig 7.14

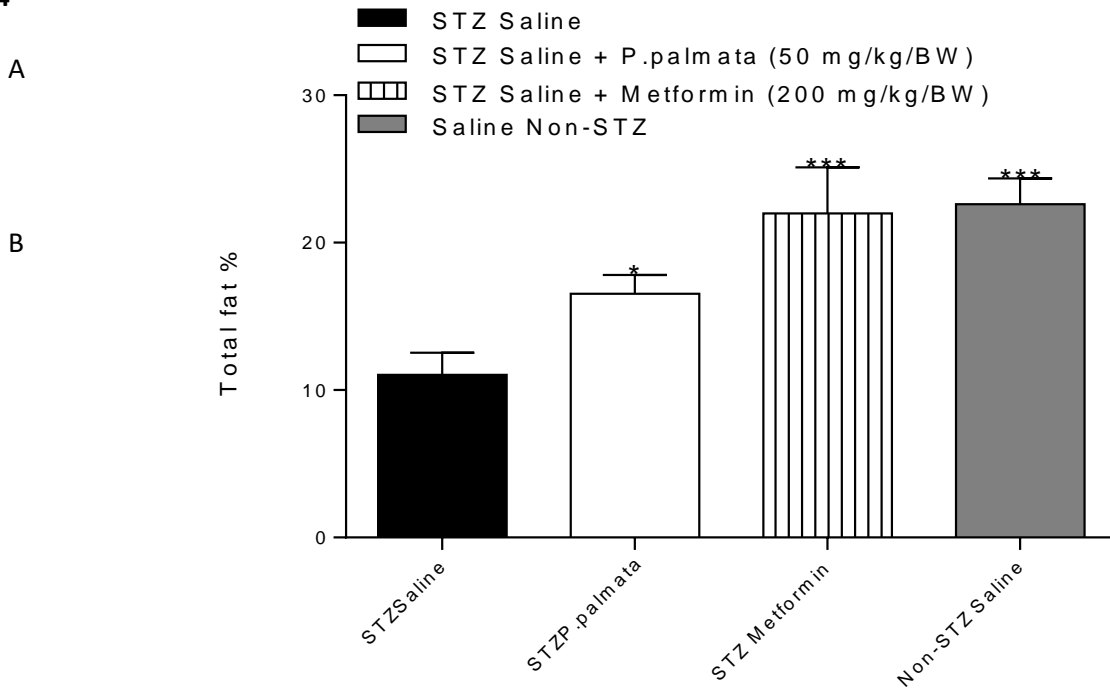


Fig 7.19 A. Effects of twice-daily administration of marine hydrolysates on anthropometric analysis assessed via PIXImus DEXA. Graph represents total body fat (%) Values represent mean \pm SEM for 7-8 mice.

7.4 Discussion

Non-fasting blood glucose analysis of STZ mice during treatment interventions

Insulin is required for carbohydrate, fat and protein metabolism at cellular level and to aid in achieving post-prandial normoglycaemia (Cornier *et al.*, 2005). In healthy individuals, the rate at which carbohydrate utilized within cellular respiration or stored as glycogen is significantly better than those subjects with type 2 diabetes (T2D) primarily due to tissue insulin resistance and diminished glucagon feedback leading to hyperglucagonaemia (Matsumoto *et al.*, 1997). Hyperglycaemia and insulin resistance promote glucagon dependent hyperglycaemia by enhancing hepatic glucose output contributing to the diabetic state (Færch *et al.*, 2016). Improvement in non-fasting blood glucose concentrations were noted within STZ mice given either the *P. palmata* hydrolysate or metformin treatment. Both *Palmaria palmata* hydrolysate and metformin caused a significant reduction in NF-BG. Improvements were noted from day 9 and throughout the study until day 18 for both PPPH treatment (day 9 $p < 0.01$ to day 18 $p < 0.001$) and metformin (day 9 $p < 0.01$ to day 18 $p < 0.001$) comparative to the saline treated STZ control group. As expected, the non-STZ mice displayed euglycaemic levels throughout the study. When analysed using the area under the curve (AUC) metformin had improved the total NF-BG compared to the hydrolysate group. In the STZ saline treated group NF-BG continued to rise throughout the study, suggesting that the relative reduction of insulin post-STZ injection was promoting sustained hyperglycaemia due to the lack of insulin present or via glucagon mediated pathways. Interestingly, the mechanism of which the hydrolysate improved NF-BG may be humoral in nature, potentially via DPP-4 inhibition seen in other studies of which improve pancreatic function via incretin hormone signalling, or gluconeogenic amino acids promoting glucagon suppression via negative feedback mechanisms (Holst *et al.*, 2017). Small molecule glucagon receptor antagonists have demonstrated excellent glucose-lowering efficacy in clinical study (Mu *et al.*, 2012). Ultimately however, the effect perhaps is contrived from a protective effect upon beta-cell survival, with results suggesting the increase in circulating insulin of which increased throughout the treatment

period would be corroborate this statement. The most potent increase in blood glucose concentration arises from hepatic glucose liberation of glycogen stores. Metformin has been shown to reduce hepatic glucose release; however, its effects are now thought to also play a key role in reducing glucose absorption from the gut. Studies have shown metformin-induced GLP-1 secretion, and as a result dual-therapy with DPP-4 inhibitors is now more common (Hundal *et al.*, 2000; Mannucci *et al.*, 2001). Indeed, metformin may well have a direct effect up plasma DPP-4 enzyme activity as demonstrated by Cuthbertson *et al.*, 2009)

Non-fasting circulating plasma insulin during treatment administration

Multiple low doses of streptozotocin (STZ) injection within the literature is used to cause mild to moderate β -cell damage via cytotoxic cellular immune response promoting hypoinsulinaemia and hyperglycaemia (McEvoy *et al.*, 1984). Typically, large doses of STZ are administered to investigate immunological changes within the pancreas more in-line with insulin dependent diabetes (Deeds *et al.*, 2011). Clinically, STZ is used as a chemotherapeutic agent in the treatment of pancreatic β -cell carcinomas, however it has been invaluable for the study of both insulin dependent and non-insulin dependent diabetes (Eleazu *et al.*, 2013). STZ has a chemical structure similar to glucose, allowing STZ to bind and enter the cell via GLUT-2 transporters (Schneidl *et al.*, 1994). STZ at lower doses elicits an immune and inflammatory reactions through release of autoantigens and macrophage infiltration. As well as the adverse effects upon glycaemia, STZ has other detrimental side effects including hepatotoxicity (Aldahmash *et al.*, 2016). Circulating plasma insulin concentrations were established from six days prior to treatment and through to day 18 of twice daily treatment administration. Initially, non-fasting insulin concentrations were reduced by approximately 75% versus that of the control mice who were not given the low dose STZ injections.. The STZ saline treated control group maintained hypoinsulinaemic state throughout the study. Compared to the latter mice, the metformin treatment group had elevated plasma insulin levels at day 6 initially, and while an increasing trend was noticed, this was not considered significant until completion of analysis at day 18. Rather

surprisingly, the hydrolysate treatment group has shown significantly higher non-fasting plasma insulin concentrations from day 6 to 18, exceptions for day 12. When assessed using insulin area under the curve (AUC), the *P. palmata* hydrolysate treatment group showed a 2-fold increase of plasma insulin while the metformin group had a 1.7-fold increase versus the STZ saline treated control group. Without intervention, all both groups of mice would thus have progressed to the severe state of hyperglycaemia similarly to the STZ saline treated group. Thus both treatment groups partially ameliorated the glycaemic decline following STZ administration. The rise in non-fasting insulin secretion is very likely linked to the improvement in non-fasting blood glucose, improvements in glucagon signalling including potentially protective effects of post-prandial GLP-1. Metformin is known to be an insulin sensitizer drug (Bailey, 2005) and indeed these mice do not display insulin resistance which could partially explain the improvement of NF-BF. Further in depth investigations on the effect on pancreatic function and improvements in circulating insulin are needed. However a study within LPS-induced RAW264.7 macrophage cells elicited anti-inflammatory effect when treated with salmon protein hydrolysates (Chang-Bum *et al.*, 2012), of which may also partly explain the improvements seen within this study.

Energy intake via dietary sources during treatment analysis

The satiety effects of proteins are well established (Westerterp-Plantenga *et al.*, 2012); within this study, the hydrolysate group caused a significant reduction in dietary energy intake. The metformin treatment group showed a food intake reduction trend, but this was deemed non-significant. From day 15, onwards the non STZ treated control animals presented a significant reduction of energy intake versus the STZ saline treated control group and similarly the hydrolysate group reduced energy intake from day 15 onwards to study completion. Hyperphagia is an expected feature of the STZ treatment process (Hidaka *et al.*, 2001). The average energy intake of the *P. palmata* hydrolysate group was 22% lower during the 18 days of hydrolysate treatment. The reason for this reduction is not related to the caloric source administered. Metformin is known for improvements in satiety, reducing food intake and improving post-prandial glycaemia (Lee & Morley, 1998). The mechanism is

not fully understood, however it may at least be partially mediated through GLP-1 or action on DPP-4 enzyme activity of which was discussed above.

Post-treatment analysis of glycated haemoglobin (HbA_{1c})

Following 18 days of various oral treatments, terminal HbA_{1c} was assessed just prior to euthanization. HbA_{1c} has become the gold standard measurement of long-term glycaemic control and used for diagnosis and prognosis of disease state in diabetes patients (Florkowski, 2013). Typically assessed at least 72 days apart in humans due to red blood cell recycling, the HbA_{1c} within the study was assessed 40 days after the first STZ injection, which included the later 18 days of treatment administration. Analysis had shown that untreated STZ saline control animals had an HbA_{1c} of 10.5% indicated a very poorly controlled glycaemic status. Interestingly, both the hydrolysate and metformin treatment groups had shown a significant reduction versus STZ saline control treatment group showing values of 7.2% for *P. palmata* hydrolysate and 6.7% for metformin. The mice not treated with STZ injections however had well controlled value of 4.05%. In relation to diabetic status, both PPPH and metformin groups would still be considered within the diabetic ranges. It is perhaps not surprising that HbA_{1c} was not reduced further, as although NF-BG was markedly reduced, however post-treatment NF-BG levels were still above 15 mmol/L. A longer treatment period may have improved both NF-BG and HbA_{1c}, however health concerns would likely be raised for the health of the STZ saline treated control if left untreated for a longer duration. Individuals with T2D who being are placed onto metformin treatment often show markedly improved HbA_{1c} within 6 months including reduction of cardiovascular events (Svensson *et al.*, 2017). Similarly, studies investigating high protein diets have shown an improvement in HbA_{1c} over the same period, however identifying the underlying mechanisms of action by which macronutrient dietary patterns are affected by increasing protein intake and how this ultimately effects HbA_{1c} is difficult to elucidate without controlling for macronutrient offset (Dong *et al.*, 2013).

Terminal excise pancreatic hormone content post-treatment

Both glycaemic and pancreatic negative feedback systems are affected throughout the progression of Type 2 diabetes (Wilcox, 2005). Within this study, we aimed to investigate total pancreatic hormone content with particular emphasis on glucagon content within the pancreas. Overall glucagon content may be significantly altered within the pancreas causing progressive hyperglycaemia and hyperglucagonemia, we aimed to assess this effect via changes in pancreatic glucagon content (Cryer, 2012). Here, 30 mg of pancreas from each animal was assessed for hormone content using the modified acid-ethanol extraction protocol (Pollock *et al.*, 1988). On analysis of glucagon, there were no significant differences in glucagon content, however the STZ saline treated control and PPPH group has displayed a tendency for reduced glucagon. Inversely however, when total pancreatic hormone content was assessed post-acid ethanol extraction, treatment groups, including the non-STZ control group displayed a 2-fold to 3-fold reduction in hormone content versus the STZ control group. When comparing the total peptide content and glucagon content, a dramatic change was noted, with 10% of the total hormone content extracted from the STZ control group arising from glucagon content, whereas the hydrolysate, metformin and non-STZ groups had over double the glucagon content in comparison to total hormone content analysed but failed to reach significance. This would support the hypothesis that within uncontrolled diabetes such as the STZ control group, glucagon content within the pancreas may be significantly depleted due to over stimulation and relative lack of insulin to promote negative feedback within α -cells. The result would further suggest that improvements seen in regards to non-fasting blood glucose are potentially at least partially derived from correction or improvement of hepatic glucose release, which is reported already with metformin.

Bone parameters and body fat and lean mass analysis after interventions in STZ mice.

Within the literature there has been inconsistency regarding the effect of diabetes on bone mineral parameters. These discrepancies described within obese and T2D individuals maybe reported due to body fat mass having impacts on the accuracy of DEXA based (Colt *et al.*, 2011). Within this study, fat

mass was not a concern, as the animals were lean and healthy prior to STZ induction of diabetes presenting weight loss due to relative lack of insulin promoting adipose utilization as a fuel source. Furthermore, associations between elevated insulin concentrations and elevated BMD are warranted, however again, this was also not a concern within the STZ groups. On analysis, there was no BMD changes within any of the treatment groups, including the non-STZ control group. Studies analysing bone mineral content (BMC) have explained that BMC may be related to bone strength and increases are not always accompanied with increased BMD which may be used to assess fracture risk (Curtis *et al.*, 2016). Rather interestingly in humans, stress fractures risk may not be protected by increased fat mass (Moayeri *et al.*, 2017). Within the present study, both the PPPH and metformin groups have shown significantly increased BMC compared to the STZ saline treated control group. In comparison to the normal mice not given STZ injections, there was no additional effect of metformin, however there was within the *P. palmata* group. Explaining the improvements in BMC may require understanding peptide-chelating properties of small molecular weight peptides (SMWPs) and amino acids. Several studies have shown that glycine is a potent calcium chelator with SMWPs similarly binding calcium to the C-terminus of the peptide chain and thought to be a viable approach for increasing the bioavailability of calcium for treatment of osteoporosis and sarcopenia in the elderly (Tang & Skibsted, 2016; Sun *et al.*, 2016; Wu *et al.*, 2017). The calcium contained within the hydrolysate after protein fractioning is currently unknown, however *Palmaria palmata* may contain 7% calcium based on dry weight, which might also explain the improvement in bone mineral content, which may show improved BMC from both peptide chelating and increased calcium absorption. Furthermore, when a more in depth analysis of a particular region of interest in the femur in the PPPH group showed enhance BMC. Each of the other groups, including the mice not injected with STZ, reported no changes in femur BMC.

Anthropometric changes

Lean mass and fat mass was reduced in each of the STZ injected groups. Lean mass was significantly higher within the mice not injected with STZ and included an increase in fat mass. As the animals progressed post-STZ injection, it is not surprising that body composition was altered, especially fat mass. The relative low levels of insulin (hypoinsulinaemia) promotes alternative utilization of adipose stores as a fuel source (Morigny *et al.*, 2016) and weight loss. The analysis of circulating lipid levels resulted in reduced triglycerides in both STZ *P. palmata* hydrolysate and metformin treatment groups including the non-STZ saline control, however this was only seen as significant within the hydrolysate group.

Conclusion

The work carried out within has been the first reported instance of improving diabetes parameters in STZ treated mice using *Palmaria palmata* protein hydrolysates. Work performed previously investigating cellular activation of cells including secretion of insulin, GLP-1, GIP, including improved glucose uptake in adipocyte cells was useful during the screening process, but was hugely dependent on the hydrolysing enzymes used. The researcher firstly identified the most promising hydrolysis method, which was screened acutely *in vitro* and *in vivo*. Following this the chronic administration of the most promising bioactive candidate within diabetic induced mice has shown improvements in non-fasting glucose, elevation of circulating insulin, lowered HbA_{1c} and improvements in lipid profiles. The results presented within, could be derived partially from the reduction of dietary energy intake, however this alone would not explain the effect upon pancreatic glucagon, anthropometric and improvement in fasting blood glucose. Interestingly, this is not the first study we have conducted with protein hydrolysates that displayed improvements in bone mineral content (Refer to chapter 6), and this represents an aspect of proteomic research that warrants further investigation for improving calcium bioavailability. With these outcomes noted, there is still unanswered questions that need addressed using a biochemical, immunological and immunohistochemistry approaches. For example, the reduction of food intake would benefit from establishing circulating satiety hormone

concentration pre- and post-treatment, including leptin, ghrelin and CCK. The research aimed to identify cellular glucagon content within the pancreas. Changes relating to improved NF-BG would most likely be derived from this mechanism partly due to only modest improvement in circulating insulin seen within treatment groups. Establishing circulating plasma glucagon levels throughout the study however, would have added to the hypothesis that the hepatic improvements of metformin, including the peptide or amino acid signalling of the hydrolysate are very likely the key factors for the antidiabetic effect presented within including a protective effect of the contained peptides within the hydrolysate on pancreatic functionality and insulin secretory effects displayed within. The research has acknowledged these approaches and aims to answer these questions in time. In relation to food derived bioactive algal protein components and sustainability of algal sources leads to the ultimate conclusion that small bioactive molecules have advantageous effects upon glucose homeostasis. The hydrolysate deployed within were produced initially at lab scale and unfortunately upscaling this approach relies mainly on the availability of the algal source including the season harvested, which significantly affects protein content, including during the processing the loss of other nutritional compounds such as fibrous, lipid and vitamin based compounds. The characterisation of bioactive peptides, previously reported to contain DPP-4 inhibitory activity (Harnedy *et al.*, 2015) may be the best approach using novel protein sources that have cultivation issues. Nevertheless, the results presented within have presented biological activity of novel small molecular weight peptides, that when taken orally improved satiety and glycaemia in a STZ diabetes induced mouse model. Ultimately, algal protein sources subjected to enzymatic hydrolysis may be used as a niche functional food ingredient with anti-diabetic properties, however further human investigations will be needed to make a stronger case.

Chapter 8

The effect of boarfish protein hydrolysate on postprandial glycaemic response and satiety in healthy adults.

Title: The effect of boarfish protein hydrolysate on postprandial glycaemic response and satiety in healthy adults.

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Contributors

Chris McLaughlin wrote the ethics for the study design. Participant recruitment was carried out by Chris McLaughlin and Dr William Crowe. Blind analysis of the blood samples were carried out by Chris McLaughlin and William Crowe. The study was unblinded by Dr Philip Allsopp once all data had been generated. The hydrolysate was generated by Padraigin Harnedy at University of Limerick and incorporation into a food stuff formulated Martin Devenny. The paper was written by William Crowe, with contributions from Chris McLaughlin, Philip Allsopp, Emeir McSorley, Finbarr O'Harte, Padraigin Harnedy and Dick Fitzgerald.

8.1 - Abstract

Background and aims

Boarfish (*Capros aper*) is a currently underutilised catch that has recently gained attention due to its potential as an alternate protein source. Furthermore, emerging *in vitro* and *in vivo* research has indicated that boarfish protein hydrolysates (BFH) may favourably impact on glycaemic control. This study aims to investigate the impact of consuming boarfish on postprandial glycaemic control in human participants.

Methods

A randomised placebo controlled crossover study was undertaken to assess the effect of consuming a drink containing BFH (3.5g) on glycaemic response in comparison to a control drink in a cohort of apparently healthy participants (n=20). Measures of satiety (ghrelin, leptin) and glycaemic control (glucose, insulin & glucagon-like peptide-1 (GLP-1)) were determined in a fasting blood sample (0 mins) and in six postprandial samples over a 180 minute period (15, 30, 60, 90, 120, and 180 mins). The participants also provided subjective measures of satiety using a visual analogue scale at each of the timepoints.

Results

A paired sample T test found no significant differences between consuming the BFH and the control drink in any of the measured blood biomarkers at any timepoint. Consumption of the BFH was shown to significantly increase satiety rating by 11.1% (P=0.034) at 180 minutes in comparison to the control.

Discussion

The study noted a lack of effect of the BFH on glycaemic control which may be due to the comparatively low dose used in this study. The satiety inducing effects of BFH needs to be further explored to determine its potential role in decreasing food intake and weight management.

8.2 - Introduction

The marine environment has historically made a considerable contribution to the human diet particularly in relation to the provision of protein and polyunsaturated fatty acids from fish (Beveridge *et al.* 2013). The existing high demand for a select range of fish species such as cod (*Gadus* genus) has led to excessive overfishing resulting in a depletion of their global stocks and has raised questions and challenges regarding their future sustainability (Srinivasan *et al.* 2010). This has led to the development of international strategies to monitor fishing of these species through the establishment of quotas and ecological monitoring programmes to avoid their endangerment (European Union, 2017). This had led to the need to increase efforts to identify alternative fish sources with a particular interest in the valorisation of the estimated that 20 million tons of marine captured fish and fish by-products that are discarded ever year (Rustad *et al.* 2003). However, it must be noted that there are considerable challenges to marketing these underutilised species due to a lack of consumer acceptability and thus many are targeted as an inexpensive source for protein extraction which is particularly important in light of the projected future challenges of

protein sustainability. Initially the isolation of protein from these underutilised fish species was for their basic nutritional properties however the development of enzyme technologies that can both recover and modify proteins resulting in the potential development of peptide hydrolysates (Kristinsson and Rasco, 2000).

The pelagic species *Capros aper* commonly known as Boarfish has emerged as a potential candidate for protein extraction as it is plentiful and inexpensive (Egerton *et al.* 2017).

Boarfish is a bycatch product of the mackerel trawl fisheries and the increased pressure from the European union to utilise unwanted by-catches has seen an increased quantity of Boarfish landing increase from 600 tonnes in 2004 to 21584 tonnes in 2008 (Common Fishing Regulation (no 2371/2002; Marine institute, 2008). Emerging evidence has suggested a role for fish derived protein hydrolysates on metabolic health (Chalamaiah *et al.* 2012). This has led to increased efforts to use novel commercial protease combinations during boarfish extraction in an effort to isolate bioactive hydrolyses for the functional food market. Previous experiments carried out by this group have demonstrated that boarfish hydrolysates have the potential to induce insulin production *in vitro* as well as improve postprandial glycaemic control in mice which has been suggested to be due to the induction of incretin hormones (e.g. GLP-1) and insulin (Parthsarathy *et al.* 2018). Furthermore, other unpublished experiments from this research group has indicated that mice consumed less food over a controlled 3 hour feeding period. Therefore, this study aims to determine if the boarfish hydrolysate can have similar effects in human participants by investigating the effect of boarfish protein hydrolysate consumption on blood glucose, insulin response and incretin hormones prior to a glucose tolerance test in human participants. The secondary aim is to determine the effect of consuming the boarfish protein hydrolysate on markers of satiety in human participants.

8.3 - Methods

Recruitment

This study was granted ethical approval by Ulster University Research Ethics Committee (UUREC) (REC/16/0078). Healthy participants were invited to participate in this is a double blinded cross over trial through email, leaflets, and posters. Participants who expressed an interest were screened for eligibility using the following criteria; non-smokers, non-pregnant or lactating, not currently suffering from any chronic illness or taking medications, aged between 18-65 years, BMI between 19.5-24.9 kg/m². All criteria was self-reported, with the exception of BMI, height was measured to the nearest 0.1cm using a stadiometer (Marsden, Leicester, UK) and weight was measured to the nearest 0.1kg using a portable Seca scale (Seca, Brosch Direct, Peterborough, UK) and BMI was calculated. Eligible participants (n=10 male, n=10 female) were asked to provide informed written consent.

Protein hydrolysate

Boarfish were caught off the coast of Ireland and supplied by Killybegs Fishermen's Organisation, Killybegs, Ireland, the boarfish used in this study were by-catches. The fish were disembowelled, minced, and stored at -20°C in Killybegs Fisheries. The protein hydrolysate was created in the University of Limerick. Details of the methodology are outlined elsewhere (Parthsarathy *et al.* 2018) however in brief, the fish meat was brought to room temperature and combined with distilled water at a ratio of 1:1 and centrifuged (Ultra-Turrax® T25 Basic, IKA®, Staufen, Germany) at 24,000 RPM/min for 15 seconds, and repeated 4 times. A 6.8 (w/v) boarfish protein suspension was obtained by adding additional distilled water. The homogenous solution was raised to 50°C and the pH adjusted to 7.0. Alcalase and flavourzyme were added at a quantity of 2.4 litres and 500 litres, respectively.

The solution was incubated at 50°C for 4 hours and 90°C for 20 minutes. The solution was double filtered (Whatman grade 1:11 µm) and freeze dried (FreeZone 18L, Labconco, MO, USA).

The dose used in this intervention study was extrapolated from our previous work that reported murine models consuming 50 mg BFH per kg body weight significantly improved glycaemic control (Parthsarathy *et al.* 2018). We estimated that human participants would weigh 70kg, and therefore the calculated dose was 3.5g of BFH.

Test drink

Due to the remaining odour and taste present in the hydrolysate, a beverage was developed to blind the treatment from both the participants and the researchers, this was achieved using a drink that contained; water, tomato, roasted pepper, chilli, and garlic. These items were cooked, blended, and stored at -20°C in Ulster University until consumption.

Study design and intervention

Twenty healthy human participants attended the human interventions suite in Ulster University on two occasions. Appointments were separated by 14 days. At the first appointment, participants were randomly assigned to consume either 60ml of the test drink with 3.5g BFH and 75g of glucose (Penlan, Kent, UK) in a 300ml solution or 60ml of the test drink without the BFH and 75g of glucose in a 300ml solution. At the second appointment, beverages were reversed.

Cannulation and sample processing

Participants were fasted overnight for 12 h on arrival. A cannula was fitted and a fasting blood sample taken (0 mins) followed by six postprandial blood samples (15, 30, 60, 90, 120, and 180 mins). Blood (10ml) was collected at each sampling point and centrifuged immediately, for 15 mins at 3000 *g*. Aliquots were stored at – 80°C until analysis.

Visual analogue scale

Participants completed a VAS whilst fasting (0 mins) and at six points postprandial (15, 30, 60, 90, 120, 180 mins). Participants ranked their hunger on a scale of 1-10 with 1 being the least hungry, and 10 being the most hungry. Participants ranked their desire to consume, sweet, salty, and savoury food on a scale of 1-10, with 1 being no desire, and 10 being a strong desire, this assessed their food preference.

Biochemical Analysis

Plasma samples collected were analysed for insulin, active GLP-1, and leptin using a multi spot electrochemiluminescent assay (Meso Scale Diagnostics, Gaithersburg, MD, USA). Inter and intra assay coefficients of variance (CV) were both below 10%. Plasma glucose was measured using the ILab 650 analyser (SpA, Milan, Italy), the CV was 1%.

Statistical analysis

Statistical analysis was conducted using statistical software GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Data was tested for normality using the Kolmogorov-Smirnov test. Variables that were not normally distributed were log transformed. Participant mean and area under the curve (AUC) blood glucose, insulin, GLP-1, ghrelin, and leptin concentrations of the BFH were compared to the control at each timepoint using a paired T test. Similarly, mean VAS scores were also compared between participants when they consumed the BFH with when they consumed the control using a paired T test. Data is presented as the mean \pm SD. A P value of < 0.05 was considered to be significant.

8.4 - Results

Participant characteristics

As shown in table 1, the mean (SD \pm) age (years) of the ten males and ten females participants was 29.20 (± 7.28), the mean (SD \pm) BMI (kg/m^2) of the participants at the treatment appointment was 19.73 (± 2.12), and at the control appointment was 19.75 kg/m^2 (± 2.09).

Blood insulin and glucose

As shown in figure 1, there was no significant difference in blood insulin concentrations between participants consuming BFH and control at any sampling point. There was no significant difference in the overall 0-180 min area under the curve (AUC) blood insulin concentration between each treatment group. Similarly, there was no significant difference

in blood glucose concentration at any sampling point between participants consuming BFH and those consuming the control. There was also no difference in the overall 0-180 min area under the curve (AUC) blood insulin concentration between each treatment group.

GLP-1

No difference in GLP-1 concentrations were observed at any sampling point between participants consuming BFH and those consuming the control (figure 2). There was also no difference in the overall 0-180 min area under the curve (AUC) GLP-1 concentration between each treatment group.

Hormone analysis

Figure 3 shows there was no significant difference in leptin concentrations at any sampling point between participants consuming BFH and those consuming the control. There was also no difference in ghrelin concentrations at any sampling point between participants consuming BFH and those consuming the control.

Satiety VAS

Participants rated their appetite and food preference on a scale of 1-10 at each of the seven sampling points. A significant increase in satiety rating by 11.1% at 180 minutes ($P=0.034$) was reported following BPH consumption in comparison to the control drink. No other differences were reported in any of the questions in the VAS.

8.5 - Discussion

This study identified that 3.5g of BFH had no effect on markers of postprandial glycaemic control. Blood glucose and insulin were not different when participants consumed boarfish protein hydrolysate compared with control. There was also no effect of BFH on ghrelin and leptin, however participants consuming the control reported they were significantly hungrier after 180 minutes compared with participants consuming the BFH.

Protein has previously been shown to impact on glycaemic control through an array of mechanisms including a potential effect on gastric emptying, a stimulation of incretin hormone release as well as a direct effect of amino acids on beta cells following absorption (Gunnarsson *et al.* 2006, Ma *et al.* 2009). However, the protein source and amino acid composition has been suggested to influence its efficacy with leucine, alanine and phenylalanine amino acids identified as potent candidates (Manders *et al.* 2012). Whey protein has been shown to be more insulinotropic than casein, gluten and cod albeit the study involved the consumption of 45g compared to the 3.5g used in the current study (Mortensen *et al.* 2009). The rapid absorption and appearance of amino acids in the blood was suggested, in part, to help explain its superiority over whey over the other protein sources, although it is clear that protein elicits its beneficial effects in a dose dependant manner (Petersen, *et al.* 2009; Akhavan *et al.* 2010). The dose selected in this study was extrapolated from murine experiments where it was deemed to be effective at modulating glycaemic control. Oral gavage of BPH (50mg/ml) in mice was shown to impact postprandial glycaemic response with a 22% reduction of iAUC blood glucose as well as an increased serum insulinemia of 36% (Unpublished observation). The BPH was suggested to modulate

postprandial glycaemia due to the presence of short peptides containing leucine, isoleucine, arginine, alanine and phenylalanine, amino acids which have previously been shown to be insulinotropic in previous studies (Manders *et al.* 2012). The lack of effect of BPH in the current study could be due to the low dose, which is supported by a study by Vikøren *et al.* (2013) where consumption of fish protein at 3g/day was shown to have no significant effect on glycaemic control over 4 weeks however a further 4 weeks at 6g/day was shown to improve glucose tolerance with a noted decrease in postprandial glucose as well as LDL cholesterol, and HDL:LDL.

The secretion of GLP-1 is known to influence glycaemic control and appetite and dietary protein has previously been shown to stimulate the release of GLP-1. It has been proposed that protein components can directly stimulate the enteroendocrine L cells to secrete GLP-1, which can subsequently bind to G-coupled receptors in β -cells resulting in cAMP mediated insulin release. *In vitro* studies previously undertaken by this research group showed that BFH significantly increased GLP-1 release GLUTag cells by 30% ($p < 0.01$) (Parthasarathy *et al.* 2018), the dosage used was similar to other *in vitro* studies (Cudennec *et al.* 2012, Harnedy *et al.* 2018). The current human study did not note any impact of consuming 3.5g of BFH on GLP-1 response which is a considerably lower dose than the study by Ma *et al.* which noted a significant increased GLP-1 status following the consumption of 55g of whey protein (Ma *et al.* 2009).

The current study found that participants consuming 3.5g BFH reported significantly less hunger compared to the participants consuming the control at 180 mins (-11.1%), albeit no difference in biological markers (ghrelin, leptin) were noted. Previous studies have shown

that protein consumption can impact on measures of appetite (Stubbs *et al.* 1996, Johnstone *et al.* 1996, Poppitt *et al.* 1998) and has been shown to be influenced by dose with higher intake (30g) increasing self reported hunger in comparison to lower intake (10g) in the absence of any alteration to ghrelin concentrations (Lejeune *et al.* 2006).

The reported appetite suppressing effects of consuming a high protein meal has also been shown to result in an increased latency time until food consumption as well as a significantly decreased food intake in comparison to either a high carbohydrate or fat meal (Chapelot and Payen 2010). It is important to note that there is evidence to implicate no role for protein content or dose on appetite and it has been shown that consumption of a meal containing 77% protein, did not change satiety, as measured by food intake, and VAS (de Graaf *et al.* 1992) and increasing protein dose had no impact. Interestingly, this study and ours, both used liquid meals, which have been suggested to be less satiating than solid meals. The satiating effects of the BFH reported in the current study will need to be followed up with BFH preload food intake study and a subsequent long term intervention to determine if BFH can impact on weight maintainance.

The current study investigated the impact of 3.5g of BFH on glycaemic control however it must be noted that whilst this dose was calculated on efficacious dose in animal models the opportunity to investigate higher doses comparable with other protein studies such as whey (55g) was limited by the considerable challenge of the sensory properties and organoleptic acceptability reported in product development. Therefore, the 3.5g of BFH per serving was deemed to avoid acceptability issues and the participants consumed the entire dose in the absence of any discomfort, albeit future development should focus efforts on modification

of the protein extraction and BFH manufacturing process to minimise/improve the sensory properties of the BFH.

8.6 - Conclusion

This study concludes that 3.5g of BFH does not affect glycaemic control. These results corroborate previous findings that have reported protein consumption results in a self-reported decrease in satiety, however no change in leptin or ghrelin. Future studies should focus on diabetic populations, utilising larger dosages of dietary protein.

Table 1: Descriptive statistics of study participants (n=20) including mean (\pm SD) biomarkers of glycaemic control and appetite

	Mean (\pm SD)
Age (y)	29.20 (7.28)
Male/Female (<i>n</i>)	20:20
BMI (Kg/m ²)	19.74 (2.11)
Insulin (mg/mL)	335.74 (367.90)
Glucose (mmol/L)	5.47 (0.44)
GLP-1 (ng/mL)	5.27 (2.10)
Leptin (ng/mL)	6.04 (10.09)
Ghrelin (μ g/mL)	309.88 (143.41)

BMI; body mass index, GLP-1; glucagon like peptide-1, SD; standard deviation. P values are generated using an unpaired T test assessing the mean difference of markers following consumption of boarfish protein hydrolysate with consumption of the control

Fig 1. (A) blood glucose, (B) insulin, (C) GLP-1, (D) leptin, and (E) ghrelin concentrations were measured in participants treated with BFH and control, at fasting, and 15, 30, 60, 90, 120, 180 mins postprandial. Values represent means \pm standard deviation. * $p < 0.05$

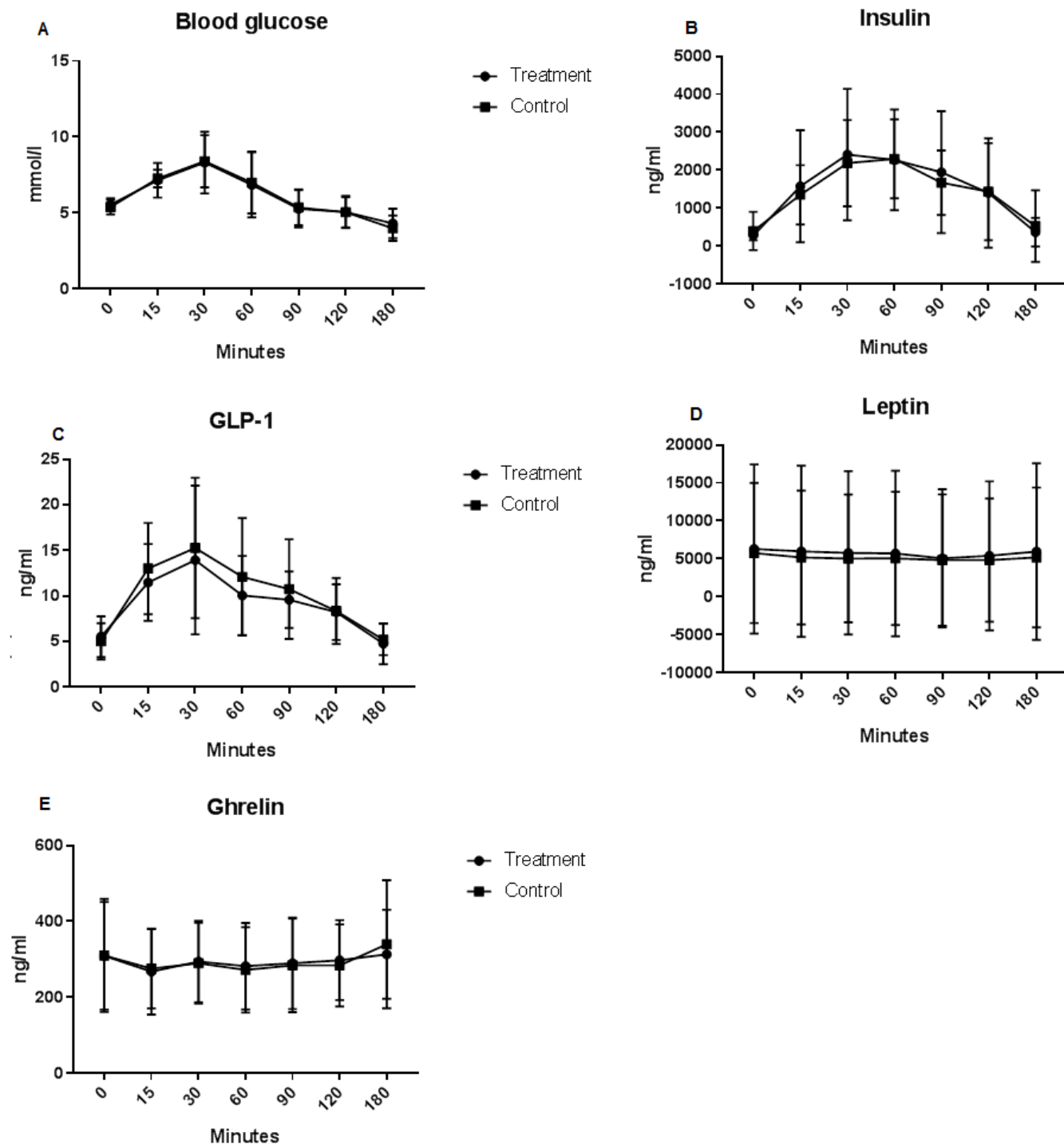
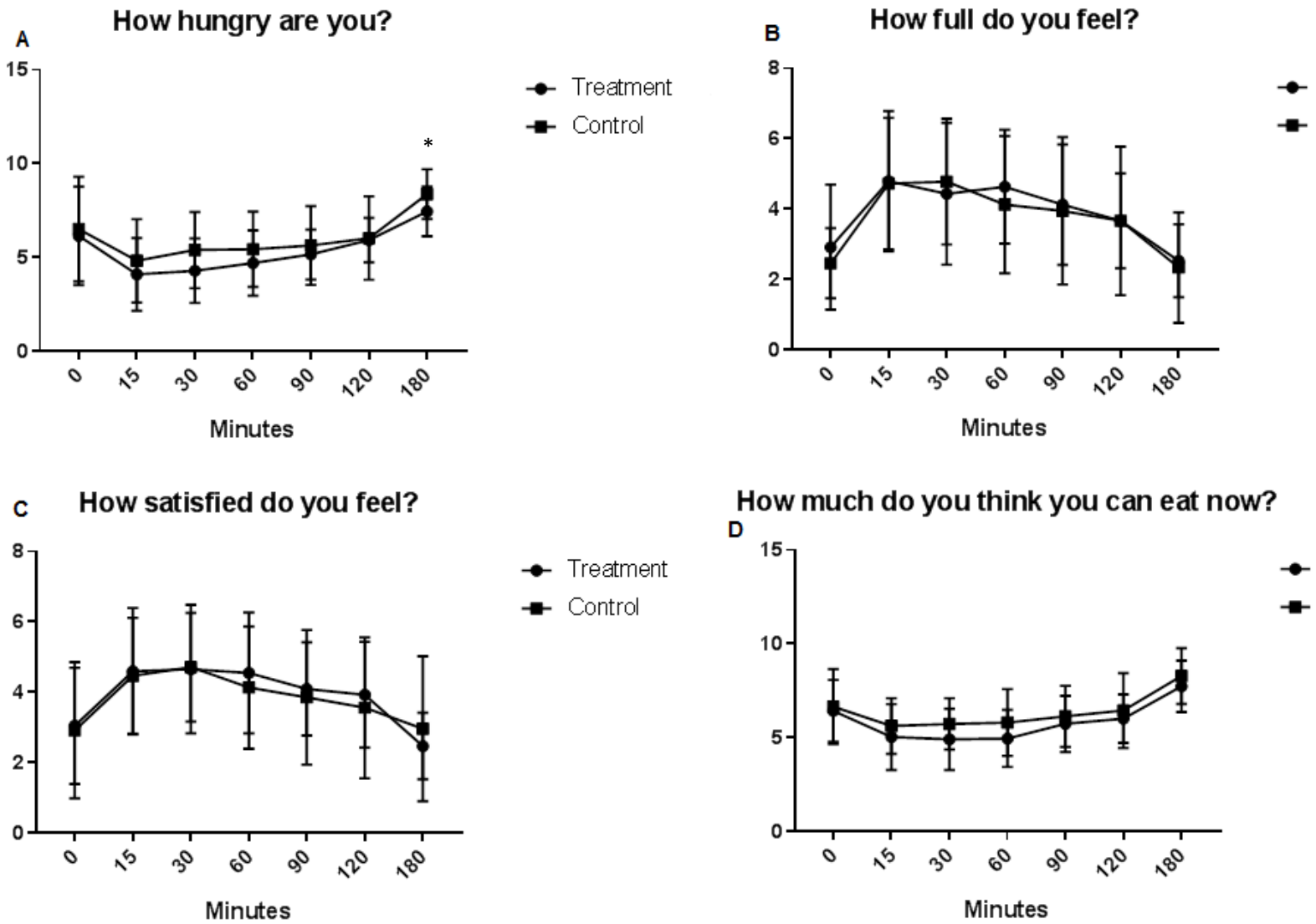


Fig 2. Visual analogue scales (VAS) were completed assessing participants self reported satiety, participants were treated with BFH and control, VAS were completed at fasting, and 15, 30, 60, 90, 120, 180 mins postprandial. Values represent means \pm standard deviation. * $p < 0.05$



Chapter 9

General Discussion

9.1 Future work approaches

The work presented within displayed the bioactivity including the oral bioavailability of small molecular weight peptides from marine sources, with emphasis on functional food development targeting obesity and type 2 diabetes. The hydrolysates analysed were derived from underutilized protein sources, including pelagic fish (Boarfish and blue whiting), discarded processing offcuts (Salmon muscle and skin) and finally a native algal source (*palmaria palmata*). The protein source was initially extracted using aqueous alkaline methods described in earlier chapters, however the generation of the small molecular weight peptides were derived from lab based enzymatic hydrolysis using singular or combinations of food grade proteolytic enzymes. To date there has been a wealth of literature additions to this area of rapidly expanding biomolecules (Giri & Oshima, 2012; Harnedy & Fitzgerald, 2012; Cheung et al, 2015)

Generated hydrolysates were first screened within cultured cells and cellular based signalling assays. This included identifying the hydrolysis method, which presented the most promising *in vitro* activity. Cell lines chosen, were tailored towards potential diabetes treatment, including insulin secreting BRIN-BD11, GLP-1 secreting GLUTag and GIP secreting STC-1 cells. Furthermore, transdifferentiated fibroblasts of which progress into adipocytes under experimental conditions were used for glucose uptake investigation in the presence and absence of insulin. It is becoming increasingly acknowledged that small molecules have the capacity to interact with large complex endogenous hormones, and as such, the research investigated protein-protein interaction of SMWPs and the ability to inhibit dipeptidyl-peptidase-4 (DPP-4) and α -amylase activity, both current treatment approaches for individuals with T2D. From here, the bioactivity was determined, including cellular signalling events. Protein, peptides and amino acids are only considered bioactive due to their cellular interaction and activation mechanics, in order to investigate this the researcher highlighted three possible approaches, including cellular membrane depolarization and secondary messenger

activation via intracellular calcium mobilization, membrane potential and cyclic AMP production during acute co-incubation with hydrolysates.

One of the most challenging aspects of orally administered amide containing biomolecules is appreciating that molecule may not remain intact in the harsh proteolytic environment of the gastrointestinal tract (GI). The hydrolysates were screened for bioactivity and were further digested using simulated gastrointestinal digestion (SGID) and were subsequently rescreened across the bioassays mentioned within. The bioactivity change was compared to the primary hydrolysate fraction, interestingly, several hydrolysates retained or improved upon bioactivity with some exceptions which will be discussed later. The oral intake of proteins and their subsequent digestion is classically investigated at the rate of which the amino acids are elevated within circulatory transport systems (Uauy et al, 2016). This approach has many limitation when administering intact proteins, and even more so when administering small molecular weight peptides. Evidence shows that small peptides have the capacity to resist further digestion and enter circulation via dependent or independent intestinal peptide transport systems (Silk, 1974).

The research aimed to highlight this important mechanism by firstly displaying the concentration of each protein hydrolysate needed to promote a biological response. The concentration ranges investigated needed to show a reduction in post-prandial glucose following an oral glucose challenge, however equally important, needed to reflect a practical protein intake quantity that could be potentially incorporated into a functional food or drink. Typically at lower doses of hydrolysates (10-25 mg/kg) resulted in no glycaemic benefit, however higher concentration ranges (50 – 100 mg/kg) had revealed significant improved when administered orally in mice. Using a combination of screening approaches, each hydrolysate had been scored on *in vitro* bioactivity including acute and delayed *in vivo* effect, and finally, the quantity needed for upscaling to human investigation was established. When considering which hydrolysate would firstly be deployed within a genetically induced diabetes animal model, two were selected, boarfish and salmon skin gelatine of

which one would be carried through to human investigation. The results presented throughout, highlighted several other hydrolysate preparations from other biological sources that were equally promising, including the algal source, however due to the limited quantity available unfortunately forced a change to a more sustainable nitrogen source.

Interestingly, each of the hydrolysates selected for chronic *in vivo* investigation had various effects upon acute and long-term glucose control, circulating lipids, and body mass and bone mineral outcomes including glycated haemoglobin improvement. Each in their own right, could have been selected for acute and chronic human study, with salmon skin gelatine being the obvious choice due to its sensory characteristics, however boarfish was selected as it was equally as promising and a readily available supply was available over the salmon counterpart.

Finally, two human intervention studies were carried out, one of which is included. Firstly, mimicking the glucose tolerance improvement displayed with boarfish, the same study approach was attempted in humans using an industry generated up-scaled hydrolysate. The lab generated boarfish hydrolysate required 50 mg/kg bodyweight to acutely elicit a post-prandial glucose improvement. This was the same dose applied to the genetically obese and diabetic (*ob/ob*) chronic animal study, however administered twice daily. Upscaling this dose from mice to humans required a 3.5 g intake for a 70 kg human or 7 g needed if following the same dosing regimen within the long-term *ob/ob* animal study. Issues had arose due to the challenging sensory characteristics of the boarfish semi-pilot hydrolysate that had been generated at industry scale. The hydrolysate unfortunately required incorporation into a food matrix to mask the sensory characteristics of the semi-pilot scale hydrolysate. The researchers collaborated with a flavour technician and chef to successful mask the sensory characteristics however no more than 3.5 g could be incorporated into the soup-based foodstuff of which was a major limitation when compared to literature doses of protein needed to see glycaemic benefit. Potentially, the lipid oxidation of the semi-pilot scale hydrolysate increased the aroma and will need to be addressed. Several improvements with the hydrolysate will be needed for

future work of which each researcher involved within the study has acknowledged, however as this was the first study of its kind including a unique approach to generating small molecular weight peptides, future study using the same approach will be much more streamlined avoiding any unsavoury issues.

Until now, this has been a brief overview of the logistics deployed within a cross-border collaborative team, used to minimise the screening time of a large range of protein products and maximise publication, industry and therapeutic potential of underutilized nitrogen sources. The remaining of the work presented within will briefly discuss key highlights of the entirety of the work looking at several key factors for obesity and diabetes treatment. These include maximizing insulinotropic and incretin secretion, targeting protein-protein interactions, satiety based improvement and finally oral bioavailability of protein molecules and the hurdles that this work overcame that future work within this area can acknowledge.

Maximising cellular secretion mechanisms of protein hydrolysates

Tailoring hydrolysates towards obesity and diabetes was the overall aim of this work. The pancreas responds well to peptide-based therapeutics, which is now well established due to the rising rate of peptide mimetics currently used to treat diabetes (Verspohl, 2009). Pancreatic islet morphology consists of diverse network of cells working in harmony to maintain glycaemia. Pancreatic cells secrete a range of glucose dependent and independent homeostatic peptide hormones including insulin, glucagon, somatostatin, amylin, pancreatic polypeptide and ghrelin. Pancreatic β -cell dysfunction plays a critical role in the pathogenesis of both type 1 and type 2 diabetes (Gromada et al, 1998). Insulin produced within β -cells is a critical regulator of metabolism and is primarily glucose responsive; however, other nutrients such as free fatty acids, peptides and amino acids can trigger potent insulinotropic responses (Torres et al, 2009). More recently, approaches exploiting insulin secretion using hormone based therapeutics such as GLP-1 and GIP have reaffirmed that peptide based insulin

secretion can be more efficient and potentially safer towards lowering elevated levels of circulating glucose (Meloni et al, 2013). Within this work, specific β -cell receptors and their activation was not investigated, however acute incubation with various hydrolysate concentrations and their effect towards general cellular signalling events were assessed. The hydrolysates were effective upon stimulating several signalling mechanisms including elevated cAMP and intracellular calcium mobilization. This would suggest that peptide-based cellular receptors were activated via the hydrolysate peptides and area which has exploded looking for the next new small peptide mimetics for improving insulin secretion. This leads to the suggestion that these hydrolysates could have other signalling effects throughout bodily systems and not necessarily confined within the pancreatic islet. An example of improving potential bioactivity was evident initially when comparing the hydrolysates generated using aqueous hydrolysis to the further simulated gastrointestinal hydrolysis (SGID) aq/alk hydrolysate. Typically, within industry, hydrolysates are generated using aqueous alkaline solutions and rarely implement enzymatic hydrolysis purely due to cost restraints. The insulin secretion data of the aq/alk hydrolysate was only elevated above basal control at 1.25mg/ml and higher, whereas further hydrolysis using SGID showed significant insulin secretion at a concentration of 0.312 mg/ml, resulting in 4 times lower dose required to stimulate a response. We hypothesize that this is primarily derived from increasing the receptor activation potential, firstly by increasing the number of generated small peptides from the intact protein source, and secondly, due to the increasing number of small molecular weight peptides a further systematic effect via multiple receptors may be possible. This was not always the case however, especially when only one proteolytic enzyme was used in the initial hydrolysis step such as the hydrolysates generated using Alcalase, Promod or bromelain only. Interestingly, the cellular secretory activity favoured hydrolysates that were generated using sequential hydrolysis of more than one proteolytic enzyme. An example of this was the hydrolysates generated using Alcalase and Flavourzyme in combination resulting in improved bioactivity with pre- and post-SGID digestion. This effect is most likely derived from both enzymes cleaving different sections of the intact protein source. Flavourzyme contains with endo- and exopeptidase activities and

can improve hydrolysis outcomes and lower the required time needed to yield small molecular weight peptides, whereas Alcalase can be further used as a serine protease targeting specific amino acids within the peptide chain further digesting larger amino acids. Finally, bioactivity of the hydrolysates generated using one proteolytic enzyme were generally improved upon once they had been subjected to SGID, this again confirms that the singular application of one enzyme may not promote the degree of hydrolysis needed to generate enough small molecular weight peptides that are under 5000 da, with the overall target from our own work showing peptides ranging from 3 to 8 amino acids having the most potent effect towards cellular activation. Excitingly, we had confirmed this after the screening of all lab based hydrolysates by further screening an industry scale hydrolysate from a common marine nitrogen source that was generated using a 45 min aqueous only hydrolysis method. The protein and peptides failed to elicit any response towards satiety or insulin secretion within cells, mainly due to the small degree of hydrolysate yield using such a short process.

Maximising hydrolysis effect and oral bioavailability

As discussed, sequential hydrolysis promoted improved bioactivity across most of the nitrogen sources investigated within. Several hydrolysates, including boarfish and *palmaria palmata* were extremely promising at improving post-prandial glycaemia, whereas salmon based hydrolysates were indeed the best candidates for reducing food intake within trained animals. Each of these mechanisms suggest that the source of protein has specific effect after hydrolysis and that disease-targeted therapeutics can be optimised dependant solely on the nitrogen source. An important factor when comparing the glycaemic versus satiety effect would be to first compare the amino acid profile of each source and determine whether there are elevation of specific amino acids within the intact protein. Salmon generated quatrapeptides containing glycine at the n-terminus have been shown to inhibit DPP-4 (Neves et al, 2017). Similarly our own work has shown quatrapeptides, pentapeptides and octapeptides having increased potency over larger peptides and proteins. The bioactivity of several sequential hydrolysis generated hydrolysates after SGID digestion was significantly reduced especially

within intestinal cell lines; however, rescreening hydrolysates after SGID may not be the best indication of oral bioactivity loss. The rationale for this comes from the length of time that the peptides were subjected to SGID, 4 h. The molecular mass screening of peptides would best suit the hydrolysis approach, aiming for hydrolysates that contain greater than 60% low molecular weight peptides may indeed increase oral bioavailability and bioactivity. Larger peptide fractions, greater than 5 – 10 kDa may not show bioactivity within the gut and certainly not within internal systems specifically due to rapid digestion by endogenous enzymes. Smaller peptides, 400 – 1200 da may be the ideal molecular weight target and one that is commonly reported within the literature. Furthermore, peptide fragments produce favourable inhibitory effects towards endogenous enzymes are also generally 4 – 10 amino acids in length rather than the penultimate amino acid residue being an indicator. This is a major issue for industry, primarily due to aqueous hydrolysis and the time required to obtain this level of consistent fragmentation and secondly, if enzymes are to be deployed the cost connected with obtaining large enzymes quantities including the additional step of enzyme denaturing, again, increases the total associated with attempting to obtain consistent SMWPs. The addition of enzymes in the right environment does have added benefits. Under experimental conditions and screening of hydrolysates, industry has the potential to accurately produce the same hydrolysates throughout consecutive runs with the aid of computer-monitored production.

Functional food development targeting gut hormones and enzymes

It would foolish to assume that all small molecular weight peptides are absorbed within the intestinal mucosa and have biological effect within internal systems, indeed we must acknowledge that the primary effect seen within our studies are derived primarily from the gastrointestinal tract itself. Peptides are extremely advantageous for cell specificity study. This was not a study outcome however, with the primary aim of producing a functional food that was firstly, inexpensive and secondly, elicited a biological response. At times screening hydrolysates within insulin secreting pancreatic cells lines may have seemed like a novel approach, especially when most diabetes pharmaceuticals have effects

upon the gut or improve upon gut hormones, however the work had shown that interaction with a complex and vital cell, such as the β -cell was indeed possible. Prior to this however, the gut and its promising approach to treating obesity and diabetes had become the primary source of bioactivity for these biopeptides as they were administered orally. It is still considered to this day that protein within the gut has minor effect until they are digested into amino acid form. Interestingly, the work within had shown significant secretion of incretin hormones, GLP-1 and GIP, and rather than the cells which secrete these hormones (L- & K-cells) being classed a nutrient sensing cells, of which they do respond to via post-prandial nutrient intake, may also be a target for small molecule activation. It will not be known however until receptor signalling work is carried out using peptides identified from the hydrolysates. The interaction of SMWPs generated from novel sources and their interaction with endogenous DPP-4 has recently evolved into the rationale that this approach can and may be a target for improvements in diabetes outcomes. Work presented in early chapters had shown that the hydrolysates generated do indeed have an inhibitory effect towards this enzyme, however improving this inhibitory activity would only be possible again, by identifying the molecules which had the inhibitory effect. With this noted, these two approach mentioned are a substantial approach to treating diabetes via improvements in incretin secretion including inhibiting DPP-4 increasing the incretin effect. This work is only within its infancy; however, future work should focus primarily on the gut and its diverse cells that can be exploited for obesity and diabetes management.

Nitrogen sources and hydrolysis approaches

Investigation of hydrolysates within this project aimed at using a stratified approach deploying each hydrolysate within the same *in vitro* and *in vivo* models. Fortunately, we can now conclude that the source of protein does indeed have varied effects, some displaying activity across a broad range of experiments while some having increased bioactivity towards a specific outcome. For example, the reduction of food intake seen within animals that were administered both versions of salmon hydrolysates would encourage future work to focus specifically on the mechanisms of which this

activity was derived using a biomarker approach. Similarly, the prolonged glycaemic improvement seen from boarfish hydrolysates or peptides from the algal source that inhibited DPP-4 with potent incretin and insulinotropic activities would warrant incorporation into a food based diet and fed to animals long-term to establish if the effect is sustained throughout obesity progression. This approach would reaffirm the bioactivity of the hydrolysates, specifically if dietary protein intake were altered with various quantities of protein from different biological sources. Most importantly however, is the hydrolysis approach to generating the hydrolysates. The molecular weight of the intact protein may be a factor when considering which method of hydrolysis more applicable, with proteins ranging from 55- to 220kDa. This suggests that lower molecular weight proteins may only require a single hydrolysis step, whereas larger proteins may benefit from sequential hydrolysis with more than one proteolytic enzyme, time will tell.

Future work considerations in brief

From the work contained within, several areas need further investigation via animal and human models of diabetes and obesity

- 1) Calcium chelating potential of small molecular weight peptides targeting individuals at risk from stress fractures
- 2) Protein hydrolysates (20g+) tested within pre- and post-diabetes individuals
- 3) The effect of protein hydrolysates on glucagon signalling in T1DM models
- 4) The DPP4 inhibitor activity via firstly pre-loading protein hydrolysates followed by a glucose challenge rather than both being administered at once
- 5) Helping industry to optimise hydrolysis profiles of protein via enzymatic hydrolysis rather than aqueous
- 6) Incorporation of hydrolysates into a food matrix at various percentages and given long-term to animals with the overall outcome targeting biomarkers of obesity and diabetes
- 7) The satiating effect of salmon protein hydrolysates

8) The delayed improvement of boarfish hydrolysates and use in protein pre-loading study

Conclusion

The work carried out over the past 3 years attempting to exploit novel and underutilized nitrogen sources has evolved exponentially not only within our own collaborative work but also across the globe. Generating novel peptides from a vast range of proteins from insects, dairy, animal, fish, macroalgae, microalgae, crustaceans and everything in between aiming to exploit therapeutic potential is now established as a viable approach to improving health; however, the nitrogen source is not always sustainable. This should not alter the approach however, as screening of biological peptides have the advantage of artificially synthesizing promising peptides that can be further modified to improve duration of action and avoid unwanted degradation once administered. This was not an approach used within this work, which aimed to reaffirm oral intake of small molecular weight peptides can have benefits beyond nutrition. This work was not without its limitations and issues, specifically sensory characteristics of marine based proteins and potentially aromatic compounds such as free fatty acids which made incorporation into a food matrix mandatory. Furthermore, translation from in vivo to human study was one of the most disappointing aspects when concluding that the nitrogen source chosen, Boarfish, may have anti-diabetic effect. The researchers have learned a viable lesson that upscaling dosages from mice to humans is not as transparent as it appears. Studies involving protein upon biological activity routinely use greater than 10g, with some as high as 50g. This was not possible due to the issues mentioned; however, production of hydrolysates will consider this with modifications already implemented to improve upon sensory characteristics. The future work should focus primarily on intestinal based hormones and improve upon bioactivity by generating hydrolysates that contain predominately peptides within the molecular weight range of 400 – 1200 da using proteolytic enzymes avoiding aqueous only hydrolysis completely. With this noted, aqueous hydrolysis is a good starting approach to initiate the breakage of peptide bonds, however the only viable approach in regards to time, is via the use of enzymes. Finally, the work presented within this

thesis has reaffirmed that novel protein sources have potent cellular secretory and signalling activity as well as acute and chronic *in vivo* improvements upon obesity and diabetes markers, with exceptions noted which would be addressed within future human investigation.

Chapter 10

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