Goat's vs Cow's Milk Consumption: Analysis of Feeding Behaviour, Brain Activation and Gene Expression in Laboratory Animals

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Abstract

Milk is a complex and highly nutritive food. In Western societies, cow's milk (CM) is most commonly consumed, but recent years have generated interest in milk from other species, especially in goat's milk (GM). Importantly, select physical and chemical properties of milk are species-dependent and – thus – so are the physiological consequences of consumption of milk sourced from specific species. For example, variation between GM and CM protein impacts digestibility and gastrointestinal processes. Consumption of GM vs CM differentially affects levels of blood hormones regulating energy balance. Furthermore, some conflicting results on acceptability of GM- and CM-based foods have been reported, and it is unclear to what extent habituation to a specific milk type underpins these parameters. To add to the confusion, CM and GM are typically consumed and, therefore, studied as modified milk products, with one of the typical compositional alterations being done to the protein fraction in which the natural 20:80 whey:casein ratio is changed to resemble the 60:40 ratio of human milk. One of the most fundamental gaps in our knowledge regarding CM vs GM relates to the acceptability, palatability and satiating properties of these milks and to appetite-controlling brain processes triggered by CM and GM consumption. Thus, in this doctoral project, I sought to examine whether GM and CM diets elicit unique feeding responses in laboratory rodents and whether the presumed appetite differences are associated with changes in neuronal activation and/or gene expression in key central regions regulating food intake.

In Specific Aim 1 of the project, I conducted a comprehensive investigation of short-term intake and palatability profiles of GM- and CM-based liquid and solid diets in mice and rats. Consumption was studied in no-choice and choice scenarios, including meal microstructure. Feeding experiments were followed by qPCR analysis of expression of relevant genes in the energy balance-related hypothalamus and brain stem, and in the nucleus accumbens, which regulates eating for palatability. I found that GM and CM are palatable to juvenile, adult, and

aged rodents. Given a choice, animals prefer GM- to CM-based diets. Analysis of meal microstructure using licking patterns points to enhanced palatability of and, possibly, greater motivation toward GM over CM. Most profound changes in gene expression after GM vs. CM were associated with the brain systems driving consumption for reward. The results allow me to conclude that, while both GM and CM are palatable, GM is preferred over CM by animals, and this preference is driven by central mechanisms controlling eating for pleasure.

In Specific Aim 2 of the thesis, I investigated the impact of whey enhancement in GM protein fraction on appetite and feeding-related brain processes. The shift from the natural whey:casein ratio of ~20:80 in animal milks is done to match the 60:40 ratio of human milk. Studies show that 20:80 versus 60:40 whey:casein milks differently affect glucose metabolism and hormone release. It is unknown whether the 20:80-to-60:40 ratio adjustment affects appetite and brain processes related to food intake. In this set of studies I focused on the impact of the 20:80 vs 60:40 whey:casein content in GM on food intake and feeding-related brain mechanisms in laboratory mice. I found that the 20:80 whey:casein GM formulation was consumed less avidly and was less preferred than the 60:40 GM in short-term choice and no-choice paradigms. The qPCR analyses in the hypothalamus and brain stem revealed that the 20:80 whey:casein GM intake upregulated genes involved in early termination of feeding and in an interplay between reward and satiety, such as MC3R, OXT, POMC and GLP1R. The 20:80 versus 60:40 whey:casein GM intake differently affected brain neuronal activation (assessed through c-Fos, an immediate-early gene product) in the nucleus of the solitary tract, area postrema, ventromedial hypothalamic nucleus and supraoptic nucleus. Overall, the findings show that whey enhancement in GM promotes overconsumption of GM in no-choice and choice scenarios and that this increased appetite for the 60:40 GM is reflected by changes in neuronal activation and gene expression relevant to feeding regulatory mechanisms.

Specific Aim 2 results showing preference for whey-enhanced GM and corresponding changes in c-Fos and gene expression, do not predetermine whether the preference for the 60:40 milk would be retained if - instead of a highly palatable GM - a somewhat less preferred CM was used. Thus, in Specific Aim 3, I replicated the aforementioned feeding, gene expression and c-Fos analyses using CM with the 20:80 vs 60:40 whey:casein. I found that mice exhibited preference for the 60:40 over 20:80 whey:casein CM. This preference for the 60:40 CM was retained even when animals had simultaneous access to the 20:80 GM. Consumption of similar quantities of 20:80 CM vs 60:40 CM differently affected c-Fos in the paraventricular, dorsomedial, arcuate and lateral hypothalamic nuclei and in the nucleus of the solitary tract in the brain stem and relative gene expression (melanocortin and opioid transcripts). It can be concluded that the 60:40 whey:casein milks are more preferred regardless of the species from which the milk was derived, indicating that whey:casein ratio influences preference. Mechanistic commonalities in the whey:casein ratio changes in CM vs GM include the hindbrain neuronal activity changes. Differences in hypothalamic c-Fos and gene expression as well as differences in no-choice feeding paradigms indicate that milk type (GM vs CM) influences some aspects of feeding processes driven by the shift in the whey:casein ratio.

Overall, the data presented in this thesis indicate that GM is generally more preferred and it has higher acceptance than CM in laboratory animal models. This phenomenon is reflected by unique changes in feeding-related brain processes induced by GM vs CM. Whey enhancement increases preference toward milk and this effect on consumption is more profound than the effect of the species from which the milk was derived. In a broader context, one has to consider, however, that whey enhancement's impact on feeding, brain activation and molecular responses might – if sustained over a longer time period - have metabolic consequences.

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List of Abbreviations

3v	Third ventricle
a-MSH	Alpha-melanocyte stimulating hormone
ActB	Beta-actin
AGRP	Agouti-related protein
AP	Area postrema
ARC	Arcuate nucelus
AUC	Area under the curve
cc	Central canal
CCK	Cholecystokinin
cDNA	Complementary DNA
CM	Cow milk
CNS	Central nervous system
cNTS	Caudal nucleus of the solitary tract
CPT1b	Carnitine palmitoyltransferase-1
Cx36	Connexin 36
DAB	3,3'-diaminobenzidine

DEPC	Diethyl pyrocarbonate
DMH	Dorsomedial hypothalamic nucleus
DMV	Dorsal motor nucleus
DNA	Deoxyribonucleic acid
GHSR	Growth hormone secretagogue receptor
GI	Gastrointestinal
GIP	Glucose dependent insulinotropic polypeptide
GLP-1	Glucagon-like protein 1
GLP-1R	Glucagon-like peptide-1 receptor
GM	Goat milk
iNTS	intermediate nucleus of the solitary tract
IR	Immunoreactivity
KO	Knockout
KOR	Kappa opioid receptor
LHA	Lateral hypothalamic area
MC3R	Melanocortin receptor 3
MC4R	Melanocortin receptor 4
MCRs	Melanocortin receptors

MOR	Mu opioid receptor
mRNA	Messenger RNA
Nacc	Nucleus accumbens
NOC	Nociceptin/orphanin FQ
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
ORL1	Nociceptin receptor
ORX	Orexin
otr	Optic tract
OXT	Oxytocin
PCR	Polymerase chain reaction
PENK	Proenkephalin
PFA	Paraformaldehyde
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
PYY	Peptide YY
qPCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid

rNTS	Rostral nucleus of the solitary tract
RT-PCR	Real time PCR
SON	Supraoptic nucleus
TBS	Tris-buffered saline
TRP	Tryptophan
VMH	Ventromedial hypothalamus
VTA	Ventral tegmental area

Chapter 1
Introduction and Aims

Food provides the necessary nutrients required for cellular respiration and function as well as for tissue growth and repair. When we are low in energy or specific nutrients, internal physiological mechanisms, including hormonal release altering brain activity, promote the sensation of hunger to encourage food intake. Following intake, the digestion and absorption of nutrients trigger alternative endocrine and neural pathways to produce satiation and attenuate feeding. If a food is particularly palatable, reward-related pathways will promote intake, even beyond the point of satiation. Foods have different compositions providing variable nutrient density, digestibility and complexity, which will inform the nature of the physiological response experienced following their intake. A foods' specific macronutrient content, nutrient bioavailability and palatability will elicit unique hormonal release influencing peripheral systems as well as stimulating disparate pathways in the brain. These pathways create the sensations of hunger, satiety and reward shaping our food intake behaviours. In this way, the food we eat can influence what and how much we eat.

Milk is a nutrient-complex food, primarily providing energy, macro- and micronutrients during infancy but which is also consumed frequently in child and adult diets in industrialised areas worldwide (Feskanich *et al.*, 2003; Drewnowski & Specter, 2004; Chevalley *et al.*, 2008; Drewnowski, 2011; Vissers *et al.*, 2011; Huth *et al.*, 2013; Cheng *et al.*, 2019). Cow's milk (CM) predominates the western milk market, accounting for approximately 82% of global milk production (Food and Agriculture Organization of the United Nations, 2018). Consequently, the majority of our understanding of the consequences of diary intake arises from studies examining CM or CM-derived products. Importantly, non-bovine milks are consumed readily in, among others, African and Asian regions, and the emerging research on unique nutritive

benefits stemming from the consumption of such milks, has generated interest in incorporating them also in the Western diet.

Goat's milk (GM), popular in areas reliant on arid agriculture, has garnered interest due to composition variations conveying unique nutritive benefits, supporting lower allergic response and easier digestion (Park, 1994; Bellioni-Businco *et al.*, 1999; Haenlein, 2007; Park & Haenlein, 2013). Despite accounting for only 2% of global milk production, the rate of growth of the dairy goat industry has surpassed that of CM production, increasing 47% between 2000 and 2018 compared to 39% growth for CM (Food and Agriculture Organization of the United Nations, 2018). With the predominant focus on CM products in nutrition research, less is known about physiological outcomes of GM consumption.

GM and CM differ in specific macronutrient composition conferring unique milk digestion kinetics and nutrient availability – notably in milk protein profiles (Ambrosoli *et al.*, 1988; Glantz *et al.*, 2010; Logan *et al.*, 2015; Maathuis *et al.*, 2017; Wendorff *et al.*, 2017; Hodgkinson *et al.*, 2018; Freitas *et al.*, 2019; Wang *et al.*, 2019; Ye *et al.*, 2019). There is also an indication of altered gastrointestinal (GI) endocrine function following GM ingestion (Rubio-Martín *et al.*, 2017). It has been shown beyond a reasonable doubt that both nutrients and hormones alter brain function to regulate feeding behaviours – however, with scarcity of GM nutritional research little is known regarding the effects of GM on feeding and central function, let alone if these are unique to that which follow CM intake.

With CM's abundance in our Western diets, GM is often perceived as novel, having a relatively "strong, smelly, salty or sweet" compared to CM with a distinct "goaty" flavour (Mowlem, 2005; Park & Haenlein, 2013). Habituation serves to confound human studies of preference and acceptance (Torrico *et al.*, 2019; Cheng *et al.*, 2020), thus the novelty of GM could influence acceptability. In basic animal research, milk often forms part of test diets utilised in analyses of feeding behaviours and physiological responses regulating intake, such as

condensed milk in obesogenic diets (Martire *et al.*, 2013; Martire *et al.*, 2014; Gomes *et al.*, 2018) and milk protein enrichment of high protein diets (Semon *et al.*, 1987; L'Heureux-Bouron *et al.*, 2004; Zapata *et al.*, 2018). Laboratory animals do not exhibit cultural diet habituation biases and they can be introduced to milk types in a controlled manner, which therefore allows for more accurate assessment of dietary interventions – including GM intake – without prior exposure influencing feeding behaviours. In addition, use of laboratory animals allows finer examination of brain function at the molecular and cellular level to define pathways involved in shaping behaviours, otherwise unachievable in human studies.

Another consideration in the acceptability of milks is, that in human diets, animal milk is rarely consumed raw. The composition of commercially available milk products is often adjusted in manufacturing, resulting in formulations with macronutrient levels unique to the original milk. Often adjustments are made to target specific consequences of milk intake, such as lowering allergenicity through eliminating lactose (Jelen & Tossavainen, 2003). The relative content of major milk proteins whey and casein are often adjusted in human milk formula diets – from the natural 20:80 whey to casein ratio to "whey-enhanced" formulations with a 60:40 ratio. The limited prior research on natural and adjusted formulations suggest this switch impacts digestion with altered proteolysis and gastric emptying rates as well as insulin-independent, hormone-mediated glycaemic control (Kung *et al.*, 2018; El Khoury *et al.*, 2019; Ye *et al.*, 2019). It would seem unsurprising that modifying whey and casein influences digestion and post-absorptive processes as, in isolation, these fractions have unique digestion kinetics (Mahe *et al.*, 1996; Boirie *et al.*, 1997; Calbet & Holst, 2004; Bowen *et al.*, 2006b; Boutrou *et al.*, 2013; Santos-Hernandez *et al.*, 2018) eliciting different endocrine response and central function (Hall *et al.*, 2003; Bowen *et al.*, 2006a; Bowen *et al.*, 2006b; Veldhorst *et al.*, 2009; Brennan *et al.*, 2012; Leidy *et al.*, 2013; Sukkar *et al.*, 2013). The peripheral response following formula intake could therefore influence feeding behaviours via modulation of central function, something observed with long term whey and casein isolate intake (Choi *et al.*, 2009; McAllan

et al., 2013; McManus *et al.*, 2015; Andreoli *et al.*, 2016; Nilaweera *et al.*, 2017). However, this is yet to described in a systematic way.

There is paucity in nutrition research concerning milk consumption and the central and behavioural changes resulting from dairy intake. This thesis will bridge some gaps in our understanding of this issue through examining feeding and brain function in laboratory rodent models following CM and GM diets. Firstly, I have examined feeding patterns of GM and CM indicative of acceptability and preference and the underlying central function supporting intake determined by analysing gene expression of relevant genes in brain pathways related to feeding regulation. Secondly, I have detailed how common changes to the relative whey and casein ratio of milk affect these behaviours and central processes. Compositional difference between GM and CM are most notable in the protein fraction, affecting both digestion and absorption processes relevant to regulation of feeding. Therefore, outcomes of modified protein in formula will be characterised in both GM- and CM-based formulations.

1.1 Milk Composition Across Goat and Cow Milks: Digestion and Absorption

Milk is a complex and compositionally 'dynamic' food which delivers all the essential nutrients for its primary function of supporting infant growth, though consumption often extends beyond infancy in adult diets. When milk is included in dietary research, or in consumer diets, there is often a simplistic generalisation of all milk being nutritional equivalent, that all milk conveys near similar outcomes in nutrition digestion, absorption and post-absorptive utilisation and response. However, milk composition varies according to species source, maternal diet and lactation period (Saarela *et al.*, 2005; Kent *et al.*, 2006; Park, 2007; Bauer & Gerss, 2011; Keikha *et al.*, 2017; Tagliazucchi *et al.*, 2018; Verduci *et al.*, 2019). Furthering this, commercially available milks are adaptations of the natural composition of raw milk through manufacturing techniques or with adjustment and addition of milk ingredients (Rudloff &

Lönnerdal, 1992; Institute of Medicine (US) Committee on the Evaluation of the Addition of Ingredients New to Infant Formula, 2004; Prosser *et al.*, 2019), thus these milks could be considered formulations. Variations in milk or milk formulation composition have notable consequences for milk digestibility and uptake (Rudloff & Lönnerdal, 1992; Lien, 2003; Wada & Lönnerdal, 2015; Prosser *et al.*, 2019). Furthermore, with milk providing the sole source of early nutrition, and as infant growth demands differ across species, milk composition also varies between different mammals (Park, 2007; Tagliazucchi *et al.*, 2018). Milk macronutrient composition between GM and CM influences consequences of milk ingestion with altered digestion, utilisation and peripheral actions – especially in the protein fraction.

1.1.1 Milk carbohydrates: Lactose and oligosaccharides

Lactose digestion products, glucose and galactose, provide energy through glycolysis and aerobic respiration or are stored glycogen utilised in hypoglycaemic states (Nordlie *et al.*, 1999). CM carbohydrate content is slightly higher at 4.6-4.9% of milk weight compared to 4.1% in GM (Jenness, 1974; Malacarne *et al.*, 2002; Park *et al.*, 2007; Park, 2010). Milk also contains oligosaccharides (reviewed in Bode (2012)), chains of monosaccharides that are indigestible and are delivered intact to the distal small intestine and colon. These promote intestinal flora growth and have bioactive properties including antimicrobial and developmental functions.

1.1.2 Milk fats

Milk fats are absorbed as free fatty acids and mono- and diglycerides in chylomicrons (Park & Haenlein, 2013). Dietary fatty acids are catabolised via beta-oxidation, providing substrates for the critic cycle and aerobic respiration (Houten & Wanders, 2010). While the lipid fraction is 3.6% and 3.8% for CM and GM respectively (Wendorff *et al.*, 2017), GM is noted for having a unique fatty acid profile conferring a characteristic “goaty” flavour (Park & Haenlein, 2013). Short- and medium-chain fatty acid content as well as smaller fat globule size potentially

increases fat-derived energy availability in GM through easier lipase activity (Attaie & Richter, 2000; Park, 2007).

1.1.3 Milk proteins

The nitrogenous fraction of milk is largely derived from proteins, predominately from whey and casein protein groups. Minor milk proteins include lactoferrin, serum albumin, immunoglobulins, hormones, enzymes, and mucins embedded in the fat globule membrane (Jensen, 1995; Park & Haenlein, 2013). Other sources of nitrogen come from non-protein nitrogen fraction including free amino acids and amino acid derivatives, urea, uric acid, and nucleotides (Jensen, 1995; Park & Haenlein, 2013). Proteinaceous digesta are absorbed as amino acids or short peptides, primarily serving as the precursors for tissue growth and repair (Gorissen & Witard, 2018). CM and GM have a similar total protein content (Park *et al.*, 2007; Wendorff *et al.*, 2017) and a 20% whey and 80% casein protein ratio (Park & Haenlein, 2013). However, they do vary in the specific proteins within the casein fraction which impacts protein digestion.

Whey proteins are soluble whilst hydrophobic caseins form micelle structures in milk solution. Whey proteins are resistant to gastric digestion, staying in the liquid component of milk digesta that is rapidly emptied into the small intestine (Jensen, 1995). Whey proteins are then digested by pancreatic enzymes and intestinal brush border membrane peptidases (Tomé & Debabbi, 1998). Casein micellar structure features α- and β-caseins precipitating with calcium phosphate, forming a colloid core surrounded by κ-casein "hairy" layer (Dalgleish, 2011). This hairy layer provides steric stabilisation in solution, however, it is susceptible to gastric proteolytic digestion and acidic conditions (Jenness, 1980). Loss of steric stabilisation leads to casein aggregation into curds which slows the rate of gastric emptying of casein proteins compared to whey (Mahe *et al.*, 1996; Boutrou *et al.*, 2013; Santos-Hernandez *et al.*, 2018). As a result, plasma amino acid appearance is rapid, with higher but transient peaks following

whey intake whereas casein intake provides slower, lower and sustained state of hyperaminoacidemia (Boirie *et al.*, 1997; Calbet & Holst, 2004; Bowen *et al.*, 2006b).

GM and CM casein content is a determining factor in each milk's digestibility. CM contains high levels of α_{s1}-casein whereas β-casein levels are higher in GM, with little to no α_{s1}-casein (Wendorff *et al.*, 2017). Rather, GM α_{s2}-casein variant is present in higher concentrations than α_{s1}-casein. Exact levels of α_{s1}-casein are genetically determined with its polymorphic gene having at least 17 variants, amongst which the D, F and G alleles provide milk with low α_{s1}-casein levels and the "null" O_1 and O_2 alleles produce no α_{s1}-casein (Grosclaude & Martin, 1997; Carillier-Jacquin *et al.*, 2016). Additionally, casein micelles in GM are larger than in CM, 260 nm (Park *et al.*, 2007) and 83-230 nm (Donnelly *et al.*, 1984; Farrell Jr *et al.*, 1990; de Kruif & Huppertz, 2012) respectively. Lower α_{s1}-casein levels and larger micelle size of GM result in looser curds during digestion (Ambrosoli *et al.*, 1988; Glantz *et al.*, 2010; Logan *et al.*, 2015; Freitas *et al.*, 2019; Wang *et al.*, 2019; Ye *et al.*, 2019). Curd density determines digestion rate as a more dense gel macrostructure limits enzyme access to protein substrates (Barbé *et al.*, 2013). Impact of this is evident with in vitro digestion of CM and GM and formula diets derived from these milks, with more rapid digestion and bioavailability of GM proteins (Maathuis *et al.*, 2017; Hodgkinson *et al.*, 2018).

Products of protein digestion are largely reflective of each milks specific protein content, i.e. CM higher in α_{s1}-casein derived peptides (Hodgkinson *et al.*, 2019). However, GM and CM formulations are comparable in protein quality, with similar ileal digestibility and digestible indispensable amino acid scores (Maathuis *et al.*, 2017). In their piglet model, Rutherfurd *et al.* (2006b) observed comparable amino acid retention between GM and CM formulations, except for higher glycine and tryptophan levels with CM. They also note adequate mineral retention with GM formula, with differences in mineral uptake again reflective of relative formula composition (Rutherfurd *et al.*, 2006a).

1.2 Milk intake, Endocrine Response and Regulation of Feeding

Milk composition alters digestion and relative bioavailability of nutrients, i.e. GM's more easily digestible fatty acid and casein fractions. Nutrient availability modifies endocrine cascades, peripheral function of the GI tract and central function of brain regions regulating feeding. There are three basic drivers of modified intake: hunger, satiety and reward. Below, I describe the general processes that underpin these three broad mechanisms at peripheral and central levels.

1.2.1 General regulation of feeding by hunger, satiety and reward

Energy intake is regulated through balanced orexigenic and anorexigenic pathways that involve disparate sets of endocrine and neural signals (Figure 1.1). In the fasted state, the enteroendocrine cells of the GI tract release ghrelin (Cowley *et al.*, 2003). Increased systemic circulation of ghrelin enhances GI motility and gastric secretions in anticipation of food intake and induces a feeling of hunger. The latter is due to interactions with ghrelin receptors, growth hormone secretagogue receptors (GHSR), expressed in the brain.

The hypothalamic arcuate nucleus (ARC) is a key feeding-related region affected by ghrelin. The blood brain barrier prevents transit of peripheral molecules from the cerebrospinal fluid directly into the brain parenchyma, thus ghrelin and other peripheral hormones act indirectly upon the ARC (Morita-Takemura & Wanaka, 2019). Communication to the ARC concerning the energy status is mediated, in part, by tanycytes. These specialised glial cells of the ependymal layer line the third ventricle allowing active transport of hormones into the brain parenchyma (Collden *et al*., 2015; Balland *et al*., 2014). Additionally, the neighbouring median eminence mediates diffusion of hormones, including ghrelin, across the blood brain barrier due to more permeable fenestrated capillaries (Schaeffer *et al*., 2013). Peripheral factors entering the median eminence, either passively diffused or actively transported via tanycytes projecting

to the median eminence, interact with projections of the ARC to modify subsequent signalling (Morita-Takemura & Wanaka, 2019).

The ARC itself contains two subpopulations of neurones that synthesise appetite regulating neuropeptides belonging to the melanocortin system (a major pathway regulating energy intake) (Sohn *et al.*, 2013). One subpopulation of ARC neurones produces pro-opiomelanocortin (POMC) which is processed into α-melanocyte stimulating hormone (α-MSH). Interactions with α-MSH and melanocortin receptors 3 and 4 (MC3R; MC4R) of the hypothalamic paraventricular nucleus (PVN) lead to activation of anorexigenic projections to the brainstem and subsequent top-down attenuation of feeding (Browning *et al.*, 2017). Countering this, ARC neuropeptide Y (NPY) and Agouti-related protein (AGRP) co-expressing neurones increase feeding through tonic inhibition of POMC neurons, antagonism of MC3/4R in the PVN and suppression of PVN neurones (Sohn *et al.*, 2013; Morton *et al.*, 2014). Ghrelin acts upon the ARC to stimulate NPY/AGRP neurones while suppressing POMC neurones, increasing feeding.

Following food intake, the presence of food in the GI tract prompts release of an alternative set of hormones promoting satiety. These hormones induce peripheral actions promoting insulin release and modulating GI function, slowing gastric emptying through the "ileal brake" mechanism that slows nutrient transit through the GI tract to maximise absorption (Van Citters & Lin, 1999). Cholecystokinin (CCK) temporarily inhibits gastric emptying and acid secretion while stimulating pancreatic and gall bladder secretion (Raybould, 2007). Glucagon-like protein 1 (GLP-1) and glucose dependent insulinotropic polypeptide (GIP) are incretins that stimulate pancreatic β cells to secrete insulin (Seino *et al.*, 2010). GLP-1 supresses glucagon release and gastric emptying, while GIP enhances glucagon release. Peptide YY (PYY) inhibits

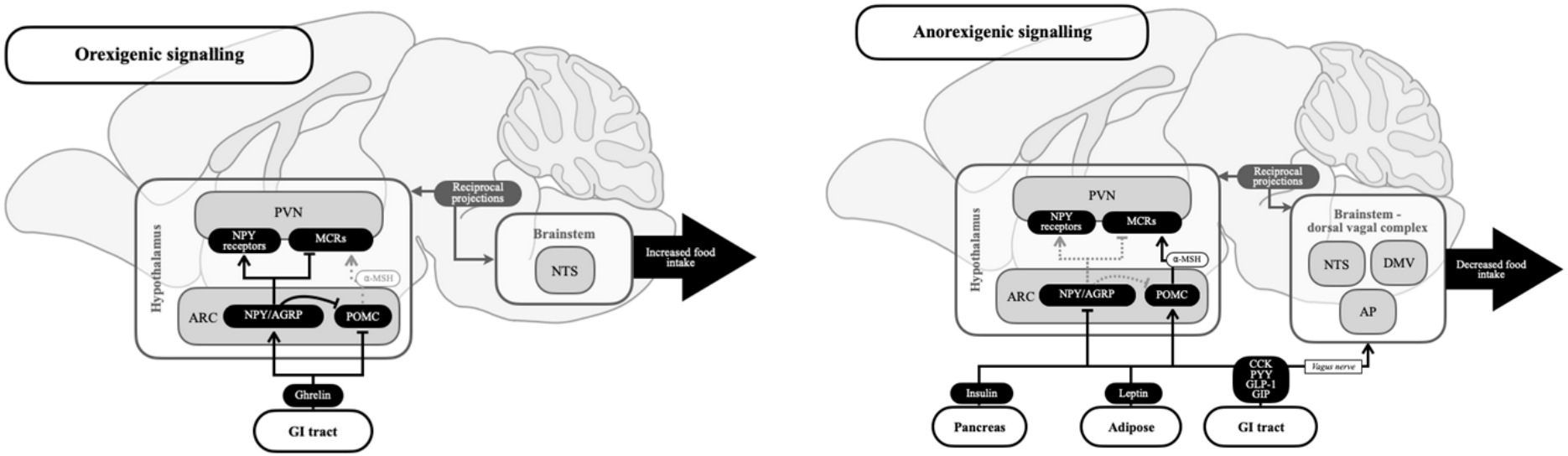

Figure 1.1: Simplified orexigenic and anorexigenic neuro-endocrine signalling pathways controlling feeding for energy. Hypothalamic arcuate nucleus (ARC) neurones express pro-opiomelanocortin (POMC), processed into α-melanocyte stimulating hormone (α-MSH). α-MSH binds melanocortin receptors (MCRs) of the paraventricular nucleus (PVN) to suppress food intake. Alternatively, ARC neurones co-expressing neuropeptide Y (NPY) and agouti-related protein (AGRP) inhibit POMC neurones and PVN activity and antagonise MC3R/4R to increase intake. The brainstems nucleus of the solitary tract (NTS) receives hypothalamic projections, mediating top-down regulation of feeding behaviours. These systems are modulated by peripheral hormones. Ghrelin released from the GI tract stimulates AGRP/NPY function and inhibits POMC to increase feeding. After food intake, the GI tract releases cholecystokinin (CCK), peptide YY (PYY), glucagon-like protein 1 (GLP-1) and glucose dependent insulinotropic polypeptide (GIP). GI hormones can stimulate vagal projections to the brainstems dorsal vagal complex, terminating in the NTS, dorsal motor nucleus (DMV) and area postrema (AP). Brainstem projections relay signals to higher brain regions – including the hypothalamus. GI hormones, insulin and leptin, secreted by adipose, act at the ARC to stimulate POMC neurones and inhibit NPY/AGRP neurones, suppressing feeding.

stomach acid secretion and emptying as well as pancreatic exocrine secretion (Takei *et al.*, 2015).

Satiation occurs with the culmination of anorexigenic post-ingestive signalling, beginning with peripheral processes of gastric distension, GI hormone release and nutrient absorption. These factors communicate status of internal milieu to the brain, subsequently altering appetite and feeding. This is mediated by vagus nerve stimulation, with stomach stretch triggering mechanoreceptors on vagal efferents while hormones and nutrients bind respective receptors (Jordi *et al.*, 2013; Browning *et al.*, 2017). These fibres project into the brainstem dorsal vagal complex, synapsing with neurones of the nucleus of the solitary tract (NTS), the dorsal motor nucleus (DMV) or area postrema (AP) and relaying status of internal milieu onto higher brain regions regulating feeding behaviours such as the hypothalamus and amygdala (Saper *et al.*, 2002; Marc *et al.*, 2014). Additionally, the circulating hormones and absorbed nutrients act at third ventricle tanycytes and the median eminence to alter brain function. CCK and GLP-1 stimulate POMC neurones (Fan *et al.*, 2004; Shah & Vella, 2014) while PYY interacts with presynaptic Y2 receptors of AGRP/NPY neurones, inhibiting their action thereby disinhibiting POMC neurones (Holzer *et al.*, 2012). Insulin, release stimulated by GLP-1 and GIP incretins, and leptin, released from adipocytes proportionally to fat mass, activates the POMC pathway and inhibits NPY/AGRP signalling (Cowley *et al.*, 2001; Begg & Woods, 2013). Hormone-mediated elevation of POMC activity leads to termination of food intake.

Reward-driven food intake is to a large extent regulated by a disparate set of pathways from the aforementioned ones that control energy intake. Intake of palatable foods, as determined by their macronutrient composition or pleasant taste (regardless of calorie content), stimulates dopamine, opioid, serotonin and endocannabinoid signalling in reward-related brain regions, from lower brainstem regions to higher-order orbitofrontal cortex, ventral palladium and nucleus accumbens (NAcc) (Berridge & Kringelbach, 2015). Reward is often separated into

three processes, “liking” (hedonic reaction to tastants), “wanting” (incentive salience, the transformation of neutral stimuli into attractive and “wanted” stimuli) and associative learning bridging the two (Berridge & Kringelbach, 2015). Opioid signalling mediates hedonic responses, with opioid signalling raises the reward value of foods (Olszewski *et al.*, 2011). Administration of opioid or receptor agonists increases “liking” behaviours in rats (tongue protrusions and paw licking). Opioid receptor agonism increases intake of palatable tastants including sugars, fats, non-caloric but sweet saccharin solutions as well as preferred caloric foods (Peciña & Berridge, 2000; Naleid *et al.*, 2007). Antagonism, commonly done in many experimental trials through non-selective receptor antagonists naloxone and naltrexone, attenuates overconsumption of palatable foods (Lynch, 1986; Olszewski *et al.*, 2011), and the anorexigenic effect is affected much more by palatability than energy density/content of a diet (Giraudo *et al.*, 1993; Levine *et al.*, 1995; Weldon *et al.*, 1996; Glass *et al.*, 2001). Dopamine signalling within the mesolimbic system, originating in the ventral tegmental area (VTA) and encompassing ventral striatal structures including the NAcc (Pandit *et al.*, 2011), produces the “wanting” aspect of reward-driven intake (Wyvell & Berridge, 2000). “Wanting”, or incentive salience, is the transformation of neutral stimuli into attractive and “wanted” ones through associative learning (Berridge & Robinson, 2003). NAcc dopamine is involved in this process, with increased dopamine in the NAcc increasing incentive salience and motivation for palatable tastants (Evans & Vaccarino, 1986; Wyvell & Berridge, 2000; Di Chiara, 2002).

1.2.2 Impact of milk consumption on signalling pathways regulating food intake

Milk intake has largely been reported to effect pathways regulating feeding for energy, whereas its effects on specific mechanisms related to reward remain less known. CM has been found to be satiating and more effective in suppressing appetite and reducing subsequent ad libitum intake than other common caloric beverages like fruit juice or soft drinks (Dove *et al.*, 2009;

Rumbold *et al.*, 2015; Onvani *et al.*, 2017). Intake is accompanied by release of GLP-1 and GIP (Maersk *et al.*, 2012), incretins that stimulate insulin release, which in turn can promote central POMC function. However, central changes following milk intake have not been described – except for serotonergic and melanocortin signalling following milk proteins, further described in Table 1.

Importantly, the alterations in endocrine signalling described above were documented following CM intake. Few studies have examined the comparative efficacy of different species milks in appetitive and physiological responses. Thus far, two studies have investigated GM and CM formulations post-absorptive endocrine response and appetitive effects. Milan *et al.* (2018) found fortified GM vs CM drinks induced similar gastric emptying rates and postprandial amino acid absorption accompanied by GLP-1, CCK, and insulin release. Participants reported no difference in hunger, fullness or desire to eat between the CM and GM diets. Appetite ratings relative to pre-intake baseline were supressed continuously for 75 minutes with the CM formulation, whereas appetite suppression after GM was found at 45 minutes then again at 90 minutes. Rubio-Martín *et al.* (2017) reported GM ingestion may support stronger satiety and suppression of hunger when compared with CM intake. Study participants were supplied with a breakfast of semi-skimmed milk, cheese – sourced from either GM or CM – with white bread. The GM breakfast reduced desire to eat and subjective hunger. This difference was potentially mediated by enhanced GI hormone release: GLP-1 area under the curve (AUC) was inversely associated with AUChunger and AUCdesire-to-eat. Considering the differences in hunger and satiety perception, potentially mediated by GI endocrine signalling, GM may elicit a unique central response in energy intake regulating brain regions.

Whilst milk nutrition research has emphasised satiety and hunger following milk intake, little has been reported regarding milk palatability or processes related to reward system function.

A tastants palatability can be derived from its nutrient composition, from one macronutrient – such as sweet sucrose solutions (Levine *et al.*, 2003), or through multiple nutrient constituents – primarily carbohydrates and fats but also proteins (Martire *et al.*, 2013; Martire *et al.*, 2014; Chaumontet *et al.*, 2018). Milk has both a high concentration of lactose and other nutrients suggesting potential for high palatability. Additionally, milk is often incorporated into rewarding diets with condensed milks in tasty, obesogenic cafeteria diets (Martire *et al.*, 2013; Martire *et al.*, 2014) or delivered alone as a highly palatable tastant (Larson *et al.*, 2002; Deacon, 2011). However, reward processes following milk have not been documented, let alone contrasted between GM and CM.

In Chapter 2 of this thesis, I have examined the differences in feeding behaviours with skim GM and CM consumption and characterise the associated central changes related to energy and reward-driven intake regulation rodent models. Systematic evaluations of short- and long-term exposure to liquid or solid diets performed accurately assess impact of different species' milk on palatability, acceptability and energy intake. With GM and CM composition altering digestion kinetics and observations by Rubio-Martín *et al.* (2017) suggesting GM may elicit different GLP-1 response and satiety level, these two milk types could alter brain function related to satiety processes. Milk also has the potential to be highly palatable, with its nutrient dense composition and prior use in rewarding diets. Therefore, central activity and gene expression in reward-related and energy regulating regions was profiled following CM and GM consumption. This was done in laboratory rodent models, eliminating habituation biases that skew human taste preferences.

1.3 Compositional changes in milk-based diets and their consequences on feeding-related processes

Whilst milk constitutes a large portion to human diets, it is rarely consumed raw (Ministry of Health, 2003; University of Otago & Ministry of Health, 2011). Milk composition is modified

during manufacturing processes or intentionally altered to exploit specific nutritive aspects of milk. For example, protein content of milks is often targeted in human milk diets to capitalise on specific health benefits – satiation of milk-based beverages or milk protein supplementation in weight management strategies (Tahavorgar *et al.*, 2014; Hector *et al.*, 2015; Verreijen *et al.*, 2015; Maher *et al.*, 2019; Rafey *et al.*, 2020), muscle development in exercise (Elliot *et al.*, 2006; Wilkinson *et al.*, 2007; Reitelseder *et al.*, 2011) and in sarcopenia (Burd *et al.*, 2012; Hidayat *et al.*, 2018) or improved immunological function, with enhanced immune response with modified milk diets (Rutherfurd-Markwick *et al.*, 2005) or antioxidant production with milk proteins intake (Parodi, 2007).

A common modification to milk proteins for human nutrition is the adjustment of the natural whey and casein profile. CM and GM naturally contain a ratio of 20:80 whey to casein (Park *et al.*, 2007; Park & Haenlein, 2013) and during manufacture whey is added to increase whey content to reach a 60:40 ratio. This is a common adjustment attempting to match human milks whey to casein ratio (Rudloff & Kunz, 1997). Whey and casein proteins are known to effect endocrine release, GI motility and absorption and central signalling in the brain – largely demonstrated in adult human and also rodent studies (Hall *et al.*, 2003; Bowen *et al.*, 2006a; Bowen *et al.*, 2006b; Choi *et al.*, 2009; Veldhorst *et al.*, 2009; Brennan *et al.*, 2012; Leidy *et al.*, 2013; McAllan *et al.*, 2013; Sukkar *et al.*, 2013; McManus *et al.*, 2015; Andreoli *et al.*, 2016; Nilaweera *et al.*, 2017). The current literature on this switch from the 20:80 whey:casein ratio to the adjusted 60:40 does suggest altered in vitro digestion, glycaemic control and post-prandial hormone release in adults. (Kung *et al.*, 2018; El Khoury *et al.*, 2019; Ye *et al.*, 2019)

1.3.1 Alteration in energy intake regulation processes with whey and casein intake

As described in Section 1.1.3, whey and casein digestion kinetics differ, with whey passing rapidly through the stomach to be quickly absorbed in the small intestine whereas casein

micelles being destabilised in the stomach, causing protein aggregation and slowing digestion and plasma amino acid absorption (Mahe *et al.*, 1996; Boutrou *et al.*, 2013; Santos-Hernandez *et al.*, 2018). Following ingestion, whey and casein elicit release of CCK, GLP-1 and PYY (Bowen *et al.*, 2006a; Bowen *et al.*, 2006b; Brennan *et al.*, 2012; Leidy *et al.*, 2013). Whey stimulates GIP release (Hall *et al.*, 2003) and – in direct comparison of whey and casein – elicits a larger secretory response of GI hormones (Hall *et al.*, 2003; Veldhorst *et al.*, 2009; Sukkar *et al.*, 2013). Potency of the endocrine response following whey intake potentially relates to digestion rate. Enhancing delivery speed of casein amino acids with a constituent free amino acid mixture, Dangin *et al.* (2001) observed more pronounced insulin release than whole casein, though not quite to the level of whey. Further slowing of casein digestion diminishes endocrine response. Juvonen *et al.* (2011) compared low-viscosity whey and viscous casein to a solid gel created by cross linking casein with transglutaminase. Pronounced peaks in insulin and CCK followed the first two diets, whereas the gel produced a lower and sustained CCK release.

Hormones released after intake of whey and casein fractions affects central processes regulating energy-driven food consumption. Consequently, whey and casein have been reported to influence relevant serotonergic and melanocortin pathways in the brain. Serotonergic function is modified with variation in central amino acid availability following whey and casein diets. Amino acids can be taken up directly into the brain across the blood brain barrier via facilitative transporters on neural capillaries membranes (Hawkins *et al.*, 2006), proportionally to dietary intake (Peters & Harper, 1985; Currie *et al.*, 1995; Choi *et al.*, 1999). Choi *et al.* (2009) examined tryptophan (TRP) availability following protein diets including casein and α-lactalbumin (a whey protein) and fluctuations in serotonin synthesis. Whey proteins do contain higher TRP content (Sindayikengera & Xia, 2006) which was reflected in serum and cortical TRP levels being higher following α-lactalbumin (Choi *et al.*, 2009). Subsequently, cortical, hypothalamic and hippocampal serotonin synthesis rates were

higher following α-lactalbumin. While serotonin is an anorexigen acting through POMC neurons (Fang *et al.*, 2013), whey's potential contributing effect to satiation remains elusive as studies on the functional link between whey intake and serotonin system's response have thus far largely focussed on the anxiolytic effect whey produces via enhanced serotonin production (Markus *et al.*, 2000; Orosco *et al.*, 2004; Scrutton *et al.*, 2007; Vekovischeva *et al.*, 2013).

Altered melanocortin signalling has only been described following long-term exposure to whey and casein isolate diets, which may explain the heterogeneity in signalling patterns observed as detailed in Table 1.

Andreoli *et al.* (2016) report central signalling changes which indicate that whey promotes satiety. Rats maintained on an obesogenic diet were transitioned onto a whey-enriched variant of the diet. This led to higher POMC expression within the hypothalamus, and it was accompanied by a reduction in food intake. Others have observed hypothalamic expression patterns that are typical of hyperphagia following high whey intake such as lowered POMC production and elevated expression of other genes that stimulate AGRP/NPY neurones. Long-term whey consumption modifies intestinal absorption capacity, with reduced intestinal weight and length and expression of glucose, fatty acid and amino acid transporters (McAllan *et al.*, 2015; McManus *et al.*, 2015; Nilaweera *et al.*, 2017; Boscaini *et al.*, 2019). This appears to elicit compensatory increases in food intake with a concurrently reduced hypothalamic POMC expression (McManus *et al.*, 2015; Nilaweera *et al.*, 2017), even when energy-dense high-sugar diets were used by Nilaweera *et al.* (2017). When these diets were reduced in energy content

Table 1: Central gene expression and physiological changes occurring with whole whey, specific whey protein or casein maintenance diets.

Study	Diet paradigm	Central changes and notable physiological changes after diet exposure
Nilaweera *et al.* (2017)	Fifteen weeks of high or low sugar diet with whey enrichment	Reduced expression of POMC, increased intake. Compensatory mechanism for reduced intestinal absorption efficiency occurring with whey consumption (McAllan *et al.*, 2015; Nilaweera *et al.*, 2017; Boscaini *et al.*, 2019). Lowering sugar content of diet exacerbated response, in addition to elevating hypothalamic ghrelin.
McManus *et al.* (2015)	Thirteen weeks of low fat, casein enriched diet or high fat diets enriched with casein or lactoferrin, a serum protein.	Lower hypothalamic POMC with lactoferrin enrichment, no changes in NPY or leptin receptor expression. Lower circulating leptin levels and increased expression of jejunal fatty acid transporters. Body weight gain was delayed with lactoferrin enrichment, however both high fat diet variants had higher bodyweight at thirteen weeks than the low-fat diet.
McAllan *et al.* (2013)	Eight weeks of high fat diet enriched with whey or casein	Reduced hypothalamic expression of insulin and leptin receptors and carnitine palmitoyltransferase-1 (CPT1b). Reduced insulin and leptin receptor expression associated with insulin and leptin resistance and development of metabolic disorders. CPT1b mediates fatty acid uptake for mitochondrial β-oxidation. Reduction in expression leads to fatty acid accumulation (Lam *et al.*, 2005), which has anorexigenic effect with central nutrient sensing, AGRP/NPY production and appetite suppression (Obici *et al.*, 2003; Lam *et al.*, 2005).
Andreoli *et al.* (2016)	Ten weeks preexposure to obesogenic phytoestrogen-free diet, followed by six weeks of phytoestrogen-free diet enriched with whey.	Increased POMC expression lowered energy intake, compared to rats maintained on phytoestrogen free diet. However, after six weeks, both phytoestrogen free diets had equivalent fat mass and body weight reductions were non-significant.

via lowering their sugar content, the orexigenic signalling patterns were exacerbated with enhanced ghrelin release (Nilaweera *et al.*, 2017). The hunger hormone ghrelin stimulates NPY and AGRP expression to increase feeding (Cowley *et al.*, 2003). McAllan *et al.* (2013) report dichotomous hypothalamic expression characteristic of both appetite stimulation and suppression. A high-fat diet enriched with whey given to rodents for eight weeks downregulated hypothalamic carnitine palmitoyltransferase-1 (CPT1b), a fatty acid transporter, but also insulin and leptin receptors transcript levels (McAllan *et al.*, 2013). Reduction of CPT1b leads to the accumulation of central fatty acids (Lam *et al.*, 2005) which prompts nutrient sensing neurones to reduced AGRP/NPY activity suppressing intake (Obici *et al.*, 2003; Lam *et al.*, 2005). However, insulin and leptin receptor suppression are of characteristic insulin and leptin resistance (Martin *et al.*, 2000; Obici *et al.*, 2002). These conditions lead to hyperphagia, dyslipidaemia and metabolic disorders including diabetes and obesity (Obici *et al.*, 2002; Grillo *et al.*, 2007; Gruzdeva *et al.*, 2019). The heterogeneity of melanocortin system function in these reports likely relates to the long-term exposure to whey or casein and the associated intestinal remodelling. Changes in central function immediately following milk protein intake are yet to be reported, though altered satiety perception with whey and casein would suggest some involvement of these systems. Whey is often observed to be more satiating than casein (Hall *et al.*, 2003; Diepvens *et al.*, 2008; Potier *et al.*, 2009; Pal *et al.*, 2014), with higher compensation in energy intake following intake (Hall *et al.*, 2003). However, others report fractions induce similar satiety (Marsset-Baglieri *et al.*, 2014) or that casein is even more effective in reducing appetite and energy intake (Abou-Samra *et al.*, 2011). Variation in appetite have also been seen with test diets that utilise the combined whey/casein fractions. Lorenzen *et al.* (2012) reported no differences in participant appetite given whey (36g), casein (34g) or skim milk (28g casein, 7g whey) drinks followed with an ad libitum lunch. However, prior milk intake lowered overall energy consumption during lunch. Diepvens

et al. (2008) examined efficacy of different protein types in shake meals in suppressing appetite, finding that the whey protein increased perceived satiety and fullness more effectively than a mix milk-protein diet (80% casein, 20% whey). Interestingly, hormone patterns observed did not support a more satiating effect of whey ingestion. The mixed milk proteins elicited higher postprandial CCK and GLP-1 response than whey alone, though whey intake produced a positive correlation with insulin and both CCK and GLP-1. Importantly, diets of with combined whey and casein fractions elicit unique endocrine activity to isolate diets (Diepvens *et al.*, 2008; Lorenzen *et al.*, 2012), which may alter central signalling systems regulating feeding given GI hormone interact with and modified select brain regions expressing relevant receptors (Cowley *et al.*, 2001; Fan *et al.*, 2004; Holzer *et al.*, 2012; Begg & Woods, 2013; Shah & Vella, 2014) and also the impact milk protein isolates have on serotonergic and melanocortin systems (Choi *et al.*, 2009; McAllan *et al.*, 2013; McManus *et al.*, 2015; Andreoli *et al.*, 2016; Nilaweera *et al.*, 2017).

1.3.2 Satiety processes effected by whey and casein adjustments

Despite extensive knowledge of how whey and casein influence satiety and hunger responses, there has been little documentation on how these processes are affected by adjustment of whey and casein ratio in milk. Given that mixed diets of milk protein produce unique effects compared to protein isolate diets, it cannot be assumed that consequences are exactly proportional to the whey or casein ratio in a diet.

Digestion kinetics and protein bioavailability of 60:40 whey:casein formulations vary from the natural ratio. Protein digestion kinetics are altered in milks with the adjusted ratio. Ye *et al.* (2019) examine in vitro digestion of proteins in both CM and GM-based formulations with the 60:40 and 20:80 whey to casein ratios. Increasing casein content increased particle size due to casein micellar aggregation. Higher casein content slowed gastric casein digestion of the 20:80

CM formula, though species differences in casein curd properties and digestion speed were noted in smaller particle size with GM formulations and equivalent hydrolysis rate between 20:80 and 60:40 GM formulations. As noted with whey and casein, speed of protein digestion and plasma amino acid appearance can impact strength of enteroendocrine cells release of GI hormones (Dangin *et al.*, 2001; Juvonen *et al.*, 2011). Another consideration in formula protein alteration is that whey addition lowers protein quality with increased the abundance of indigestible protein products following thermal manufacturing process. Pasteurization and ultra-high temperature processing, intended to reduce milks microbial load to extend shelf life, can alter milk protein structure and bioavailability. The tertiary structure of whey proteins is easily destabilised with heat and form novel aggregates after thermal treatment (Jean *et al.*, 2006; Patel *et al.*, 2006). Additionally, Maillard reactions of proteins glycating with reducing sugars block proteins lysine residues, limiting proteolytic action on glycation products and lowering protein availability (Wada & Lönnerdal, 2015; van Lieshout *et al.*, 2020). Indigestible products remain intact through the GI tract (Sillner *et al.*, 2019; Sillner *et al.*, 2020) promoting growth of aberrant microflora (Seiquer *et al.*, 2014; Bui *et al.*, 2020). Added whey in formula diets increases the abundance of these glycation products (Prosser *et al.*, 2019), potentially reducing digestibility. Proteins that are more easily digested and more readily absorbed – such as pre-digested hydrolysates (Calbet & Holst, 2004; Diepvens *et al.*, 2008; Koopman *et al.*, 2009)– enhance hormonal release and stronger satiety.

Changes in post-prandial glycaemia were reported in studies by Kung *et al.* (2018) and El Khoury *et al.* (2019) with milk drinks with modified protein ratios that were consumed alongside high carbohydrate cereal breakfast (Kung *et al.*, 2018; El Khoury *et al.*, 2019). Healthy adult participants were given high- or low-protein (3.1% and 9.3% of weight respectively) milk formulations with either 20:80 or whey-added 60:40 whey to casein ratios. All treatments reduced blood glucose before ad libitum pizza lunch, however intake was not

modified. Whey to casein ratio affected pre-lunch blood glucose, with a lower peak with the 60:40 formulation and reduced appetite with the 20:80 milk (Kung *et al.*, 2018). Changes in blood glucose were insulin independent, with treatments not eliciting changes in insulin or c-peptide levels (El Khoury *et al.*, 2019). Rather, elevated hormonal responses included elevated premeal GLP-1 with the high protein concentration and 60:40 ratio as well as enhanced pre-meal CCK release with high protein formulations and lowered ghrelin post-meal with the 60:40 ratio. The authors suggest that gastric emptying speed, modified by post-prandial hormones, determined the rate of carbohydrate intestinal delivery and absorption to produce the changes in blood glucose response.

Investigations of protein in infant milk formula – which is heavily modified both in total content and specific protein composition to attempt to match breastfed infant growth and metabolism – have found negligible effects of this switch with similar growth patterns across infancy (Grant *et al.*, 2005; Koletzko *et al.*, 2009; Weber *et al.*, 2014; Zhou *et al.*, 2014; Gruszfeld *et al.*, 2016; Totzauer *et al.*, 2018). Janas *et al.* (1987) reported that formulations with 18:82 or 34:66 whey to casein ratio not only produced similar anthropomorphic measures but also similar plasma amino acid fluctuations. Importantly, these studies span across infancy, not detailing variation in immediate feeding responses.

These changes in digestion and endocrine response with varying relative whey and casein content are unsurprising when considering that these processes are uniquely modified following whey and casein isolate intake. However, unlike literature concerning these milk protein isolates, effects on satiety and hunger central control systems has not been investigated. El Khoury *et al.* (2019) noted altered release of GI hormones GLP-1 and CCK, which, as described in section 1.2.1, typically stimulate POMC pathways, promoting satiety. Interestingly, milk diets with varied whey and casein content have not been reported to modify

appetite perception, despite difference in acute whey and casein satiety response and variation that comes with mixed milk protein intake. Kung *et al.* (2018) and El Khoury *et al.* (2019) do not report differences in appetite response following the modified milk and cereal breakfast despite changes in hormones known to regulate appetite (Kung *et al.*, 2018; El Khoury *et al.*, 2019). They suggest satiety to be confounded by intestinal discomfort with high lactose content of milks or the meal's high energy content. How adjustments to whey and casein ratios in milk formulations has not been accurately assessed, nor is it known how post-prandial endocrine release impacts related feeding behaviours.

1.3.3 Impacts of modified protein content on milk product palatability

Aside from feeding related to energy status, foods can be consumed for their palatability. Modifications made to milk composition are reported to impact milk product taste, however, this is limited to flavour assaying or side observations in larger studies of physiological parameters with little understanding of how feeding or reward-related central activity is impacted.

Whey and casein content have been reported to influence the palatability of milk products. Flavour assaying of yoghurts by Tomaschunas *et al.* (2012) indicated increased protein content and lower casein content produced less flavourful products with a yellowy appearance and grainier texture. These poorer sensory attributes were mitigated by increasing fat content. Cheng *et al.* (2019) similarly assessed sensory properties of increased protein and variable milk serum proteins (whey and other soluble minor milk proteins) and casein content in milk beverages. Similarly, increased serum protein concentration increased aroma, sweet aromatic, cooked and sulphur and cardboard/doughy flavours and yellowness. Increased casein content increased drink viscosity. Potier *et al.* (2009) gave women "cheesy" snacks with casein alone or a mixed snack with 66:33 whey to casein. While this study focussed more so on the energy

compensation following intake, with reduced intake of an ad libitum lunch and in total daily energy intake following snacks, authors also note participants rated the mixed snack as more palatable with better taste, texture and appearance. Variability in sensory attributes across ranges of macronutrient concentrations – including whey and casein content – emphasises the need for balancing composition to ensure diet acceptability.

Altered intake and palatability of isolated factions and mixed diets are also noted in animal models, specifically in obese rats utilised by Pezeshki *et al.* (2015). Feeding patterns and metabolic consequences of long-term consumption of whey, casein or mixed milk protein diets were unique, with intake, body weight and fat mass declining more rapidly on isolate diets. This could in part due to alterations in circulating hormones, with the whey diet producing higher GLP-1 levels and insulin sensitivity than the mixed diets, but also due to relative palatability of isolate versus mixed protein diets. When diets were simultaneously presented with a lower protein control diet containing only 14% protein energy from egg white, consumption of mixed diet was near that of the control and significantly lowered across early timepoints for both isolate diets, indicating mixed protein had iso-palatability with the control whilst isolate diets were less palatable.

Diet palatability varies across milk formulations, milk product and diets with variable whey, casein or mixed milk protein content. Importantly, palatability of milk formulations with adjustment of whey and casein content impacts flavour. However, it is unknown how the reported sensory attributes influence the control of feeding behaviours. Relative palatability of mixed protein diets and isolate diets appears relevant in animal modelling, impacting energy intake and contributing to alterations in body composition (Pezeshki *et al.*, 2015). Thus, adjusting relative whey and casein content could impact palatability, and therefore acceptability, of milk formulations.

With the modifications in endocrine release following milk formulations of variable whey and casein content – and changes to melanocortin and serotonergic function following whey and casein isolates – it would not be surprising if altering the relative ratios of whey and casein in milk diets would impact central circuitry regulating intake for energy. Additionally, adjusting milk protein content impacts the palatability of milk diets, which could alter processes underlying feeding for reward. Thus, in understanding physiological outcomes in the switch from the natural 20:80 whey to casein ratio to the adjust 60:40 ratio, it would seem obvious to investigate if acute feeding behaviours are modified by changing whey:casein ratios and what central pathways underly these responses. In Chapters 3 and 4 I address this line of enquiry. Acceptability and preference for milk formulations containing the natural 20:80 and whey-enhanced 60:40 ratios were examined in adult mouse models. Expanding upon this, the accompanying changes in activity and expression of relevant genes in energy intake-regulating regions of the brain were also described. These experiments were performed with both GM and CM derived-formulations. The species difference in whey and casein profiles, digestive properties and initial reports of modified post-absorptive hormonal response indicated that responses to modified whey and casein content could be altered dependent on milk source.

1.4 Aims

The **overarching aim** of this doctoral research was to examine whether milk-based diets derived from GM or CM elicit unique feeding patterns in rodent models and whether differences in appetite response are mediated by central processes regulating intake. This project encompassed three aims:

Specific aim one: To determine whether GM and CM based diets (liquid and solid chow) produced variable appetitive behaviours of acceptance and preference in mouse and rat models. To examine whether behaviours are accompanied by changes in expression of key genes associated with hedonic and homeostatic feeding regulation within relevant brain regions controlling intake (Chapter 2).

Specific aim two: To determine whether adjustment of milk protein content (ratio of whey and casein) of GM-based infant formulations modify acute feeding behaviours (acceptance and preference) in the mouse model. To examine whether changes in intake of diets with variable protein content are accompanied by changes in neuronal activation (c-Fos) and gene expression of key regulatory genes in brain regions associated with appetite regulation (Chapter 3).

Specific aim three: To determine whether adjustment of protein content (ratio of whey and casein) of CM-based infant formulations modify acute feeding behaviours (acceptance and preference) and whether adjustments to protein content alter interspecies milk diet preferences in the mouse model. To examine if divergent feeding patterns between CM formulations are accompanied by changes in neuronal activation and gene expression of key regulatory genes in brain regions associated with appetite regulation (Chapter 4).

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Chapter 2

Palatability of goat's versus cow's milk: insights from the analysis of eating behaviour and gene expression in the appetite-relevant brain circuits in laboratory animal models

2.1 Abstract

Goat's (GM) and cow's milk (CM) are dietary alternatives with select health benefits shown in human and animal studies. Surprisingly, no systematic analysis of palatability or preference for GM vs. CM has been performed to date. Here, I present a comprehensive investigation of short-term intake and palatability profiles of GM and CM in laboratory mice and rats. Consumption was studied in no-choice and choice scenarios, including meal microstructure, and by using isocaloric milks and milk-enriched solid diets. Feeding results are accompanied by qPCR data of relevant genes in the energy balance-related hypothalamus and brain stem, and in the nucleus accumbens, which regulates eating for palatability. GM and CM were found palatable by juvenile, adult, and aged rodents. Given a choice, animals prefer GM- to CM-based diets. Analysis of meal microstructure using licking patterns points to enhanced palatability of and, possibly, greater motivation toward GM over CM. Most profound changes in gene expression after GM vs. CM were associated with the brain systems driving consumption for reward. It is concluded that, while both GM and CM are palatable, GM is preferred over CM by laboratory animals, and this preference is driven by central mechanisms controlling eating for pleasure.

2.2 Introduction

Milk is a widely consumed, affordable, and highly nutritive food, which serves as a key source of, among others, protein, calcium, potassium, magnesium, and vitamins (especially A and D) in industrialised countries (Feskanich *et al.*, 2003; Drewnowski & Specter, 2004; Chevalley *et al.*, 2008; Drewnowski, 2011; Vissers *et al.*, 2011; Huth *et al.*, 2013). In Western societies, cow's milk (CM) products represent the largest share of dairy available on the market, and cow's skim milk varieties have become common. However, recent years have generated interest in milk from other species, such as goat's milk (GM). The use of GM as an alternative to CM has been driven by the findings in humans and laboratory animals showing potential beneficial nutritive consequences of GM intake and differences in physiological responses to GM or CM consumption, (for review, see (Haenlein, 2007)). For example, Bellioni-Businco *et al.* (1999) reported that individuals with a CM allergy were able to drink five times more GM than CM before the symptoms of an allergic response appeared (Bellioni-Businco *et al.*, 1999). In studies utilising rodent models, Barrionuevo et al. demonstrated that GM increases utilization of copper, zinc, and selenium (Barrionuevo *et al.*, 2003). Bioavailability of iron and copper was found to be improved in GM-fed rodents suffering from malabsorptive syndrome and in healthy controls (Barrionuevo *et al.*, 2002; Barrionuevo *et al.*, 2003). Finally, GM improved bone turnover in iron-deficient rats compared to rats fed CM (Diaz-Castro *et al.*, 2011; Diaz-Castro *et al.*, 2012).

Surprisingly, little is known about GM's acceptance and preference relative to the main dairy product in today's food environment in the Western world. There is no systematic analysis of propensity to ingest GM and CM or relative palatability of GM vs. CM in either humans or in laboratory animal models. Consequently, our understanding of acceptance and palatability of GM compared to CM is still mainly based on anecdotal evidence and on market availability, both heavily influenced by local cultural or environmental aspects (such as in Western vs.

Asian countries) and habituation-driven intake of a specific milk type (Silanikove *et al.*, 2010). This is a major gap in knowledge as palatability affects, among others, the amount of food eaten in a single meal, the rate of consumption, food anticipation, and satiety. It has a profound effect on activity of brain circuits responsible for processing energy intake (including the hypothalamus and brain stem) and reward (such as the nucleus accumbens; NAcc) (Olszewski *et al.*, 2008; Gosnell & Levine, 2009; Olszewski *et al.*, 2011). These parameters can, in turn, impact a plethora of mechanisms outside the central nervous system (CNS), via neural and hormonal interactions linking the brain and peripheral tissues (Agusti *et al.*, 2018; de Kloet & Herman, 2018; Schwartz, 2018).

Here, I present a comprehensive investigation of short-term intake and palatability profiles of GM and CM in laboratory rodent models (mice and rats) using skim milks. Consumption data details the acceptance (no-choice) and preference (choice) scenarios of calorie-matched milks and milk-enriched solid diets. Consumption data are accompanied by the analysis of expression of appetite-related genes in the hypothalamus and brainstem, two brain regions predominantly involved in energy balance control, and in the NAcc, a key site that regulates eating for palatability (Olszewski *et al.*, 2008; Olszewski *et al.*, 2011). Relative mRNA levels of genes involved in promoting consumption, such as those encoding neuropeptide Y (NPY), Agouti-related protein (AGRP), ghrelin receptor, orexin, opioid peptides/receptors, and gap junction protein, connexin 36 (Cx36), were analysed via RT-qPCR. The analysis also included transcripts related to decreased appetite and termination of consumption, such as oxytocin, melanocortin receptors 3 (MC3R) and 4 (MC4R), and proopiomelanocortin (POMC). Typically, presentation of tastants that differ in palatability and composition, among other traits, evokes some changes in expression within this subset of genes, reflecting a different propensity of an animal to ingest specific diets (Olszewski *et al.*, 2008; Olszewski *et al.*, 2009). A number of physiological functions by the brain vary with age, including appetite. Weight is

typically gained throughout early and middle age, followed by gradual, age-associated anorexia. In line with that, a drive to consume food (and responsiveness to palatability) is high during the earlier stages of life, whereas in aged animals, anhedonia and decreased responsiveness to rewarding diets and to drugs that promote eating for pleasure ensue (e.g., see (Gosnell *et al.*, 1983; Morley, 2013; Zink *et al.*, 2014)). Therefore, in the following feeding experiments, rodents belonging to three distinct age groups: adolescents, adults, and aged animals, were used. It should also be noted that rats and most mammals, other than select groups of humans, poorly digest lactose post-weaning. Though lactase activity in adult rats is residual, rats fed as much as 30% lactose in their daily diet from post-weaning to day 98 had normal body growth or body weight course (their body weight was somewhat lower) (van de Heijning *et al.*, 2015). However, in this current study focused on short-term rather than long-term exposure to milk or milk containing chow, minimising this impact.

2.3 Material and Methods

2.3.1 Animals

Male Sprague-Dawley rats and C57Bl mice (all weaned on day 21) used in these studies were single-housed in a temperature-controlled (22 °C) animal facility with a 12:12-h LD cycle (lights on at 07:00). Standard chow (Diet 86, Sharpes Stock Feed, Wairarapa, New Zealand) and water were available ad libitum unless indicated otherwise. The University of Waikato animal ethics committee had approved the procedures (ethics approval numbers: 1020, 1043, and 1057), and they are compliant with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publ., no. 80–23, rev. 1996). Feeding experiments were performed in separate cohorts of animals (weight-matched) unless specified otherwise. The age of animals included in the adolescent (5–6 weeks), adult (3–5 months), and aged (25–27 months) categories was based on previous publications pertaining to the aging process in rodents (McCutcheon & Marinelli, 2009). It should be noted that despite poor digestibility of lactose post-weaning, no signs of gastrointestinal discomfort or sickness were observed, which is in line with previous studies showing that rats fed as much as 30% lactose in their daily diet (thus, more than given here) for several weeks displayed good tolerance of the carbohydrate (van de Heijning *et al.*, 2015).

2.3.2 Skim milk diets

Milk diets (Dairy Goat Cooperative, Hamilton, New Zealand) were stored as powder and prepared immediately before use by being reconstituted in water. Composition of the milks are shown in Table 2.1. GM- or CM-enriched chows (Dairy Goat Cooperative, Hamilton, New Zealand) were refrigerated and brought to room temperature prior to administration. See appendix 6.1 for composition of GM and CM chow.

Table 2.1 CM and GM milk powder composition

	Protein	Fat	Carbohydrate	Ash	Moisture
CM	37.1	1.1	51	6.5	4.3
GM	36.1	0.9	49.9	9.5	3.6

2.3.3 Feeding studies

Diet treatments given either species' adult, adolescent and aged cohorts and the energy status (sated/deprived) are outlined in Table 2.2.

Table 2.2 Schematic of skim milk diet treatment paradigms across age, energy status and species

	 Sated	Deprived	 Adult	Adolescent	Aged	 Rat	Mouse
Single tastant							
Skim milks + sucrose + cornstarch	X		X	X	X	X	X
		X	X			X	X
Skim milk chows	X		X			X	X
		X	X			X	X
Choice							
Skim milk 2 bottle choice	X		X	X	X	X	X
Skim milk chow choice	X		X		X	X	X
Skim milk lickometer	X		X			X	
Chow choice over 72 hr	X					X	

2.3.3.1 Episodic Intake of Individually Presented GM and CM in Sated Adult Mice and Rats

Protocol were based on previous studies assessing episodic intake of palatable tastants (Olszewski *et al.*, 2010; Herisson *et al.*, 2014; Herisson *et al.*, 2016). Individually housed mice and rats were accustomed (in homecages) to receiving one of the four isocaloric (0.6 kcal/g) solutions for 2 h/day on 2 days (10:00–12:00) prior to the experiments using their usual 250 mL sized water bottles (used for all bottle scenarios) to avoid neophobia(mice: n = 8–9/group;

rats: 8–10/group): GM, CM, an energy-equivalent 15% sucrose solution (a reference palatable solution), or a 15% cornstarch suspension (a negative control for palatability; as cornstarch is insoluble in water, 0.3% xanthan gum was added to this liquid in this experiment as described previously in (Bonacchi *et al.*, 2010)). On the experimental day, bottles with the solutions (at room temperature) were placed in the cages and water and chow were removed for the 2-h experimental session. Spillage (g) from each individual bottle was recorded before placement into cage. Intakes were measured after 2 h using a digital scale and expressed in grams per gram of body weight. This feeding experiment was conducted in a separate cohort of animals.

2.3.3.2 Energy Deprivation-Induced Intake of Individually Presented GM and CM in Mice and Rats

Mice and rats previously exposed in their homecages to GM, CM, cornstarch, and sucrose were deprived of standard chow overnight (food taken away at 16:00). On the next day (10:00), water bottles were removed and replaced with bottles (at room temperature) containing one of the four treatments (mice: n = 8–10/group; rats: 7–8/group). Spillage (g) from each individual bottle was recorded before placement into cage. Intakes were measured using a digital scale after 2 h and expressed in grams per gram of body weight. This feeding experiment was conducted in a separate cohort of animals.

2.3.3.3 Episodic Intake of Individually Presented GM- and CM-Enriched Chow in Sated Adult Mice and Rats

Rats and mice were given episodic access to the chow enriched with GM or CM according to the protocol described above, where, instead of GM or CM, a GM- or CM-enriched chow was presented for 2 h (10:00). Standard chow pellets were removed during this 2-h meal, but water was left in the cages. Intake of chow pellets (at room temperature) was measured using a digital scale after 2 h and expressed in grams per gram of body weight. In order to assess baseline

intake, a control group of animals had a fresh batch of the standard chow placed in the hopper for 2 h (n = 7–8/group for both mice and rats). This feeding experiment was conducted in a separate cohort of animals.

2.3.3.4 Energy Deprivation-Induced Intake of Individually Presented GM- and CM-Enriched Chow in Adult Mice and Rats

Rats and mice previously exposed to GM- and CM-enriched chow (pre-exposure to both chow types was simultaneous) were deprived of standard chow overnight (food taken away at 16:00). On the next day (10:00), animals received either standard chow, GM- or CM-enriched pellets (mice: n = 7–8/group; rats: n = 8–9/group) at room temperature. Intakes were measured using a digital scale after 2 h and expressed in grams per gram of body weight. This feeding experiment was conducted in a separate cohort of animals.

2.3.3.5 Episodic Intake of Individually Presented GM and CM in Sated Adolescent and Aged Rodents

Mice and rats aged 5–6 weeks (n = 9–11/group for each species) were used in the study on adolescent animals, whereas 25-month old mice and 26-month old rats (n = 8–9/group for each species) were used as the aged cohorts. The feeding experiments utilising individually presented cornstarch, sucrose, GM, and CM solutions followed the protocol described above for the relevant studies in adult sated rodents that received one of the four solutions for 2 h. This feeding experiment was conducted in a separate cohort of animals.

2.3.3.6 Episodic Intake of GM and CM Presented Simultaneously in Sated Adolescent, Adult, and Aged Rodents

Mice (n = 20) and rats (n = 21) aged 5–6 weeks were used in the study on adolescent animals, 16–18-week old mice (n = 10) and rats (n = 12) were included in the study on adults, whereas

25-month old mice (n = 12) and 26-month old rats (n = 11) were used as the aged cohorts. Adult and adolescent rats and mice had been previously exposed to GM and CM (pre-exposure to both milk types was simultaneous). The aged animals came from the cohorts described above in section 2.3.3.5, however, a week-long 'washout' period was allowed between the previous experiment and this study. First, the animals were accustomed to simultaneously receiving GM and CM as a two-bottle choice (bottles placed next to each other; random order) for circa 1 h per day on two days in their homecage. Then, on the experimental day, chow and water were removed from cages and GM and CM (at room temperature) were given to the animals for 2 h (11:00–13:00). Spillage (g) from each individual bottle was recorded before placement into cage. Intakes were measured using a digital scale after 2 h and expressed in grams per gram of body weight. This feeding experiment was conducted in a separate cohort of adolescent and adult animals.

2.3.3.7 Episodic Intake of GM- and CM-Enriched Chow Presented Simultaneously in Sated Adult and Aged Rodents

Mice and rats aged 18–20 weeks old mice (n = 8) and rats (n = 8) were included in the study on adults, whereas 25-month old mice (n = 9) and 27-month old rats (n = 10) were used as the aged cohorts. Adult rats and mice had been previously exposed to GM and CM chow (pre-exposure to both chow types was simultaneous). The aged animals came from the same cohort as in section 2.3.3.5, again with a two-week-long washout period. First, the animals were accustomed to receiving simultaneously CM- and GM-enriched chow in a subdivided hopper in their homecages (placement of GM/CM pellets was random; standard chow was removed) for ~1 h per day on two days. Then, on the experimental day, after removal of standard chow, CM- and GM-enriched pellets (at room temperature) were given to the animals for 2 h (10:00–12:00). Intakes were measured using a digital scale after 2 h and expressed in grams per gram of body weight. This feeding experiment was conducted in a separate cohort of adult animals.

2.3.3.8 Lickometer-Assessed Preference for Simultaneously Presented GM and CM in Sated Adult Rats

Six 12-week old male rats were housed individually in cages equipped with bottles attached to lickometers (Lafayette Instruments, Lafayette, IN, USA). The animals were previously given GM and CM to prevent neophobia (the pre-exposure was simultaneous). They were accustomed to receiving a choice between GM and CM on two separate days for 30 min (random order of bottles) in lickometer cages. On the experimental day, standard chow and water were removed from the cages and animals were given simultaneous access to GM and CM (room temperature) for 30 min. The number of licks on each bottle was counted and analysed (Scurry Activity Monitoring software, Lafayette, IN, USA), both as total number of licks as well as number of licks per 5-minute interval. We also assessed the cluster number (number of bouts of licking—each bout was defined as continuous licking interspaced by no more than 0.5 s between each other) and an average cluster length (bout duration measured in seconds) of GM vs. CM. This feeding experiment was conducted in a separate cohort of animals.

2.3.3.9 72-h Cumulative Intake of Simultaneously Presented CM- and GM-Enriched Chow in Adult Rats

First, the animals were accustomed to receiving two types of chow pellets (room temperature) simultaneously in a subdivided hopper in their homecage (placement of pellets was random) for circa 2 h per day on two days. On the experimental day 1 (17:00), animals received a choice of either standard/CM chow (n = 9), standard/GM chow (n = 10), or GM/CM chow for 72 h (pellets were exchanged daily; n = 16). Cumulative 72-h intakes were recorded in grams. This feeding experiment was conducted in a separate cohort of animals.

2.3.4 Effect of 24-h CM vs. GM Consumption on Feeding-Related Gene Expression in the Brain Circuit

In order to assess the effect of 24-h intake of GM and CM solutions on the expression of feeding-related genes in the brain, mice were given CM or GM (at room temperature) as the only tastant (starting at 10:00). Animals given water served as baseline controls. At 10:00 on the subsequent day (thus, 24 h after milk presentation), the animals were sacrificed via cervical dislocation. Brains were dissected out and the hypothalamus, NAcc, and brain stem excised and stored in RNAlater at −80 ° C until further processing. This experiment was conducted in a separate cohort of animals.

Tissues were homogenised in Trizol (Ambien), 1 mL per 0.1 g tissue. 0.2 mL chloroform was added and samples were centrifuged at room temperature for 10 min at 10,000× *g*. The clear phase containing RNA was isolated and 0.5 mL of isopropanol was added. RNA was precipitated in an ice bath for 10 min then centrifuged at 4 °C for 20 min at 10,000× *g*. Aqueous phase was removed from the pellets, which were then resuspended in 0.3 mL of ethanol and centrifuged at 4 °C for 10 min at 10,000× *g*. Liquid was removed and pellets were air-dried.

Pellets were dissolved in 8 µL DEPC water and 1 µL DNAse buffer (dNature). Samples were then incubated with 1 µL DNAse (dNature) at 37 °C for 30 min. DNAse was inactivated via addition of stop buffer (dNature) and incubation at 67 °C for 10 min. Removal of DNA was confirmed via PCR using HOT FIREPol Blend Master Mix (dNature), followed with agarose gel electrophoresis. Concentrations of RNA were measured with a nanodrop.

cDNA was synthesised from RNA samples with iScript Advanced cDNA synthesis kit (BioRad). Synthesis of cDNA was confirmed with PCR followed by agarose gel electrophoresis. Quantitative RT-PCR (qPCR) was used to determine relative expression levels

of housekeeping genes (ActB, GAPDH, β-tubulin) and of genes of interest. Reactions contained 4 µL of 25 ng/µL sample cDNA, 1 µL of each forward and reverse primers (5 µM), 10 µL iTaq Universal SYBR Green Supermix (BioRad) and 4 µL MilliQ water. qPCR experiments were performed in duplicates alongside negative controls of MilliQ water for each primer pair. Amplification protocol was initiated at 95 °C for 15 min, followed by 45 cycles of 15 s at 95 °C, 15 s at the primer-specific annealing temperature and 30 s at 72 °C. Primers used are detailed in Table 2.3.

2.3.5 Data Analysis

Analyses of qPCR data utilised BioRad CFX Manager software (BioRad); one-way ANOVA followed by Bonferroni's test with the correction for multiple comparisons was used, with $p < 0.05$ set as criterion of statistical significance. Feeding data from studies comparing two groups were analysed using a t-test, whereas comparisons between three or more groups were done with ANOVA followed by Bonferroni's post-hoc test, with differences considered significant when $p < 0.05$.

Table 2.3 Forward and reverse primers for housekeeping and target genes used in RT-qPCR analyses of hypothalamic, brainstem and NAcc relative gene expression following GM and CM consumption

Gene	Forward	Reverse
GADPH	5′-AAGGTCATCCCAGAGCTGAA-3′	5′-CTGCTTCACCACCTTCTTGA-3′
βTUB	5′-CGGAAGGAGGCGGAGAGC-3′	5′-AGGGTGCCCATGCCAGAGC-3′
ActB	5′- GTGTGACGTTGACATCCGT-3′	5′-TGCTAGGAGCCAGAGCAGTA-3′
POMC	5′-CCTTGTGGGTCTGTTTGA-3′	5′-AGCAGCCTCCCGAGACA-3′
AGRP	5′-GGATCTGTTGCAGGAGGCTCAG-3′	5′-TGAAGAAGCGGCAGTAGCACGT-3′
NPY	5′-GGTCTTCAAGCCGAGTTCTG-3′	5′-AACCTCATCACCAGGCAGAG-3′
MC4R	5′-CTTATGATGATCCCAACCCG-3′	5′-GTAGCTCCTTGCTTGCATCC-3′
GHSR	5′-TCCGATCTGCTCATCTTCCT-3′	5′-GGAAGCAGATGGCGAAGTAG-3′
ORX	5′-GCCGTCTCTACGAACTGTTGC-3′	5′-CGCTTTCCCAGAGTCAGGATA-3′
OXT	5′-CCTACAGCGGATCTCAGACTG-3′	5′-TCAGAGCCAGTAAGCCAAGCA-3′
OXTR	5′-TCTTCTTCGTGCAGATGTGG-3′	5′-CCTTCAGGTACCGAGCAGAG-3′
PENK	5′-CGACATCAATTTCCTGGCGT-3′	5′-AGATCCTTGCAGGTCTCCCA-3′
DYN	5′-GACAGGAGAGGAAGCAGA-3′	5′-AGCAGCACACAAGTCACC-3′
MOR	5′-CCTGCCGCTCTTCTCTGG-3′	5′-CGGACTCGGTAGGCTGTAAC-3′
KOR	5′-CACCTTGCTGATCCCAAAC-3′	5′-TTCCCAAGTCACCGTCAG-3′
PNOC	5′-AGCACCTGAAGAGAATGCCG-3′	5′-CATCTCGCACTTGCACCAAG-3′
ORL1	5′-ATGACTAGGCGTGGACCTGC-3′	5′- GATGGGCTCTGTGGACTGACA-3′
GLP1R	5′-ATGGCCAGCACCCCAAGCCTCC-3′	5′-TCAGCTGTAGGAACTCTGG-3′
Cx36	5′-CCAGTAAGGAGACAGAACCAGAT-3′	5′-GATGATGTAGAAGCGGGAGATAC-3′

2.4 Results

In the non-choice acceptance tests, sated adult mice and rats showed very low levels of consumption of a 'bland' cornstarch emulsion, whereas intakes of the GM (mice, $F(3,30) = 62.8$, $p < 0.001$; rats, $F(3,32) = 25.5$, $p < 0.001$) and CM (mice, $p < 0.001$; rats, $p < 0.001$), as well as of the sucrose solution (mice, $p < 0.001$; rats, $p < 0.001$), were several times higher than of cornstarch. Energy-deprived animals had a higher baseline intake of cornstarch, but consumed significantly more sucrose (mice, $F(3,32) = 9.77$, $p \leq 0.001$; rats, $F(3,26) = 5.5$, $p = 0.039$), GM (mice, $p < 0.001$; rats, $p = 0.0023$), and CM (mice, $p = 0.034$; rats, $p = 0.0083$; Figure 2.1 A–D). Similarly, both deprived and sated adult individuals ate more GM- and CM-enriched pellets than standard chow (sated mice: $F(2,19) = 5.9$, GM, $p = 0.029$ and CM, $p = 0.011$; sated rats: $F(2,19) = 20.5$, GM, $p < 0.001$ and CM, $p = 0.0011$; deprived mice: $F(2,19) = 6.5$, GM, $p = 0.0058$ and CM, $p = 0.034$; deprived rats: $F(2,22) = 10.8$, GM, $p < 0.001$ and CM, $p = 0.0442$; Figure 2.1E–H). Adolescent and aged sated mice and rats (Figure 2.2A,B,E,F) given episodic 2-h access to one of the solutions, consumed more GM (adolescent mice, $F(3,35) = 42.7$, $p < 0.001$; rats, $F(3,36) = 16.9$, $p < 0.001$; aged mice, $F(3,29) = 31.2$, $p < 0.001$; rats, $F(3,29) = 18.9$, $p < 0.001$), CM (adolescent mice, $p < 0.001$; rats, $p < 0.001$; aged mice, $p < 0.001$; rats, $p < 0.001$) and sucrose (adolescent mice, $p < 0.001$; rats, $p < 0.001$; aged mice, $p < 0.001$; rats, $p < 0.001$) than cornstarch.

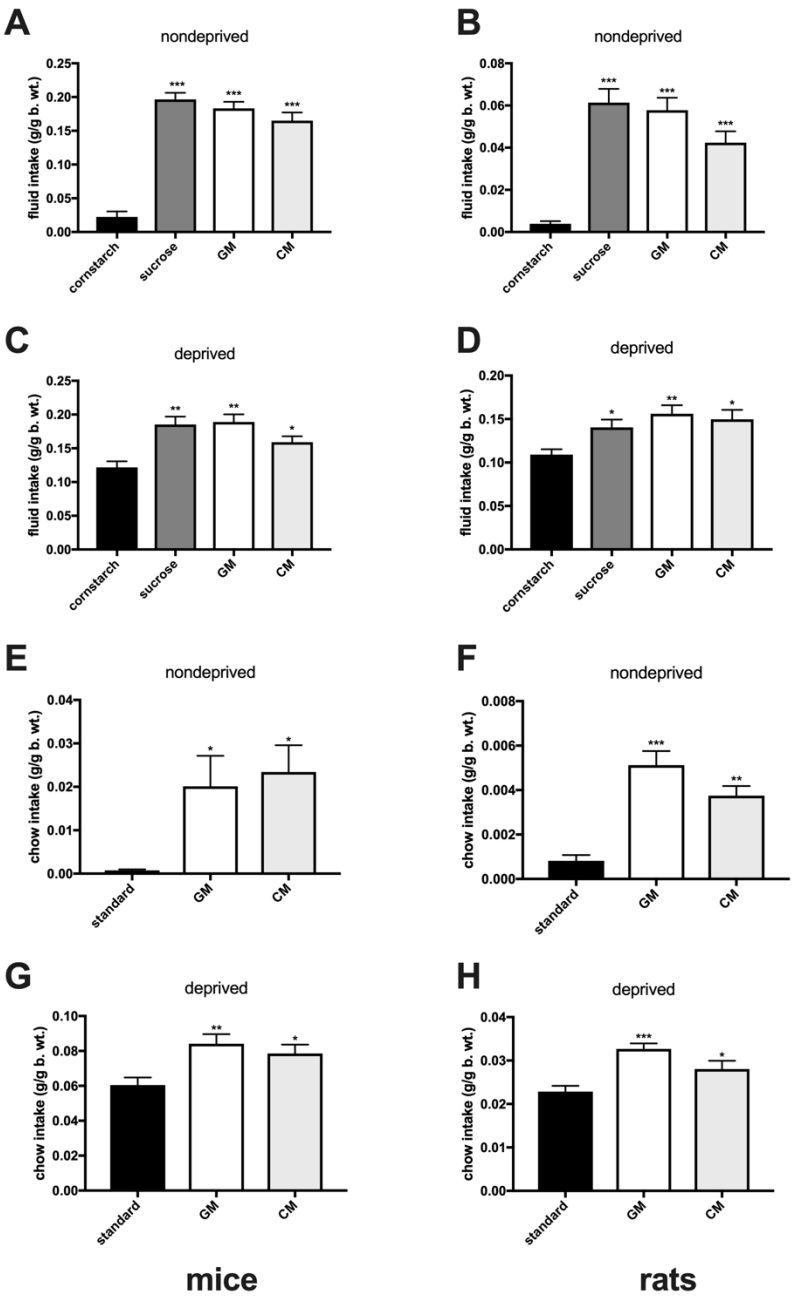

Figure 2.1 Episodic 2-h consumption of individually presented (acceptance) cornstarch, sucrose, GM, and CM isocaloric solutions (**A**–**D**), and of standard, GM- and CM-enriched chow (**E**–**H**) in sated (nondeprived) and energy-deprived mice (left panel) and rats (right panel).*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

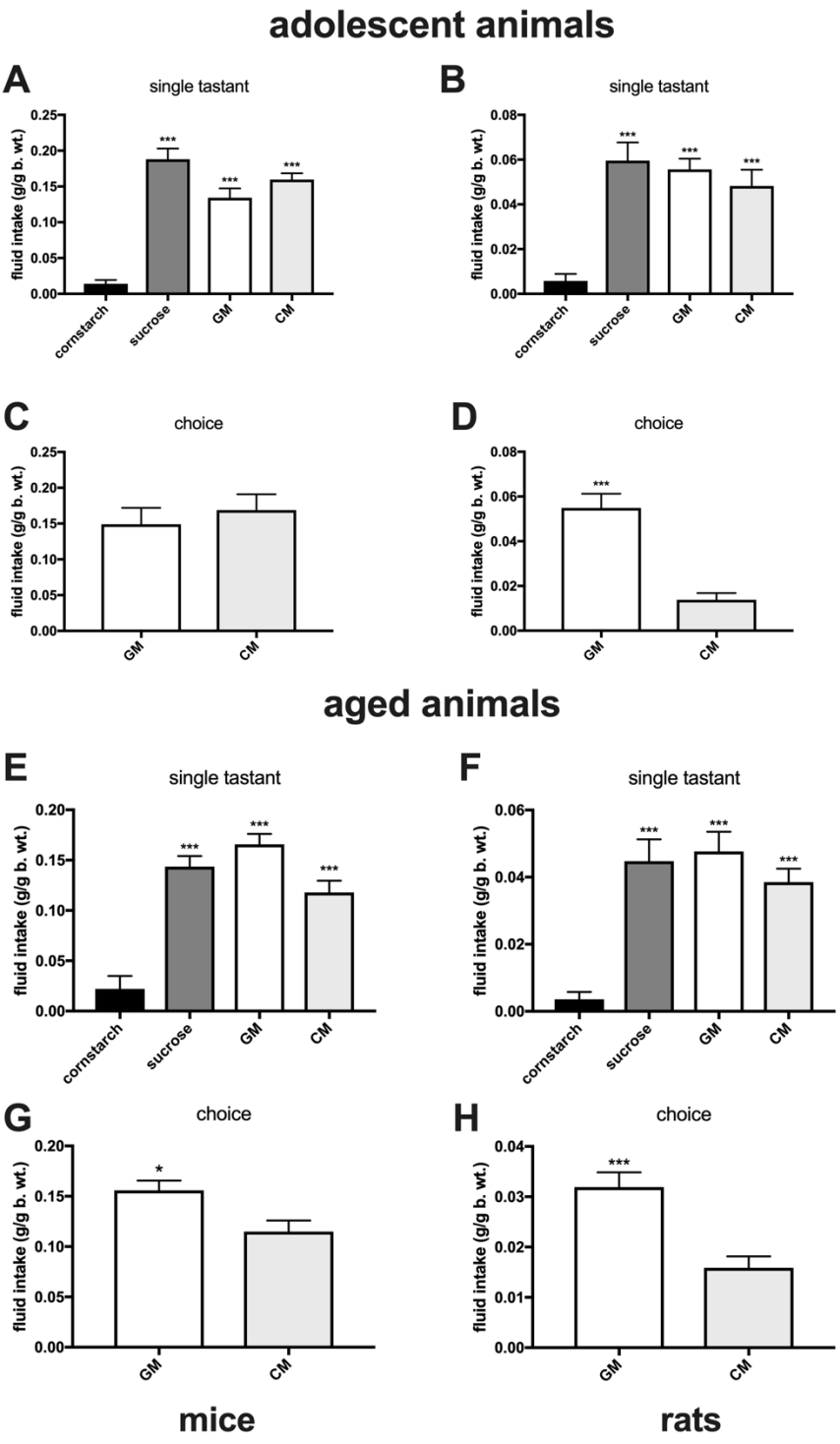

Figure 2.2: Episodic 2-h consumption of individually presented cornstarch, sucrose, GM, and CM isocaloric solutions (**A**,**B**,**E**,**F**: acceptance) and simultaneously given GM and CM (**C**,**D**,**G**,**H**: preference) in adolescent and aged sated mice (left panel) and rats (right panel). *, $p \leq 0.05$; ***, $p \leq 0.001$.

When given a 2-h episodic choice between GM and CM, all age cohorts of rats (adolescent, $p < 0.001$; adult, $p < 0.001$; aged, $p < 0.001$) and adult and aged mice ($p = 0.012$ and 0.011, respectively) preferred GM (Figure 2.2C,D,G,H and Figure 2.3A,B). During a brief, 30-min exposure to both GM and CM in cages equipped with lickometers, adult rats exhibited a more robust response to GM cumulatively over that period ($p = 0.01$) as well as during the first ($p = 0.037$) and second ($p = 0.05$) 5-min time interval of the meal (Figure 2.3C,D). There was a trend approaching significance ($p = 0.088$) toward an increase in the cluster number (number of licking bouts) of GM over CM, and a significantly greater cluster length of each GM than CM bout ($p = 0.022$; Figure 2.3E,F). In choice experiments involving GM- and CM-enriched chow, adult and aged rats ($p = 0.009$ and 0.023, respectively) and adult mice ($p = 0.028$) preferred GM chow, whereas in aged mice, a trend toward GM preference was detected ($p = 0.059$) (Figure 2.4A,B). Adult rats given a 72-h uninterrupted access to a choice between GM and CM chow preferred GM chow ($p < 0.001$), while both GM ($p = 0.015$) and CM pellets ($p < 0.001$) were preferred over standard food during a similar time of exposure (Figure 2.4C).

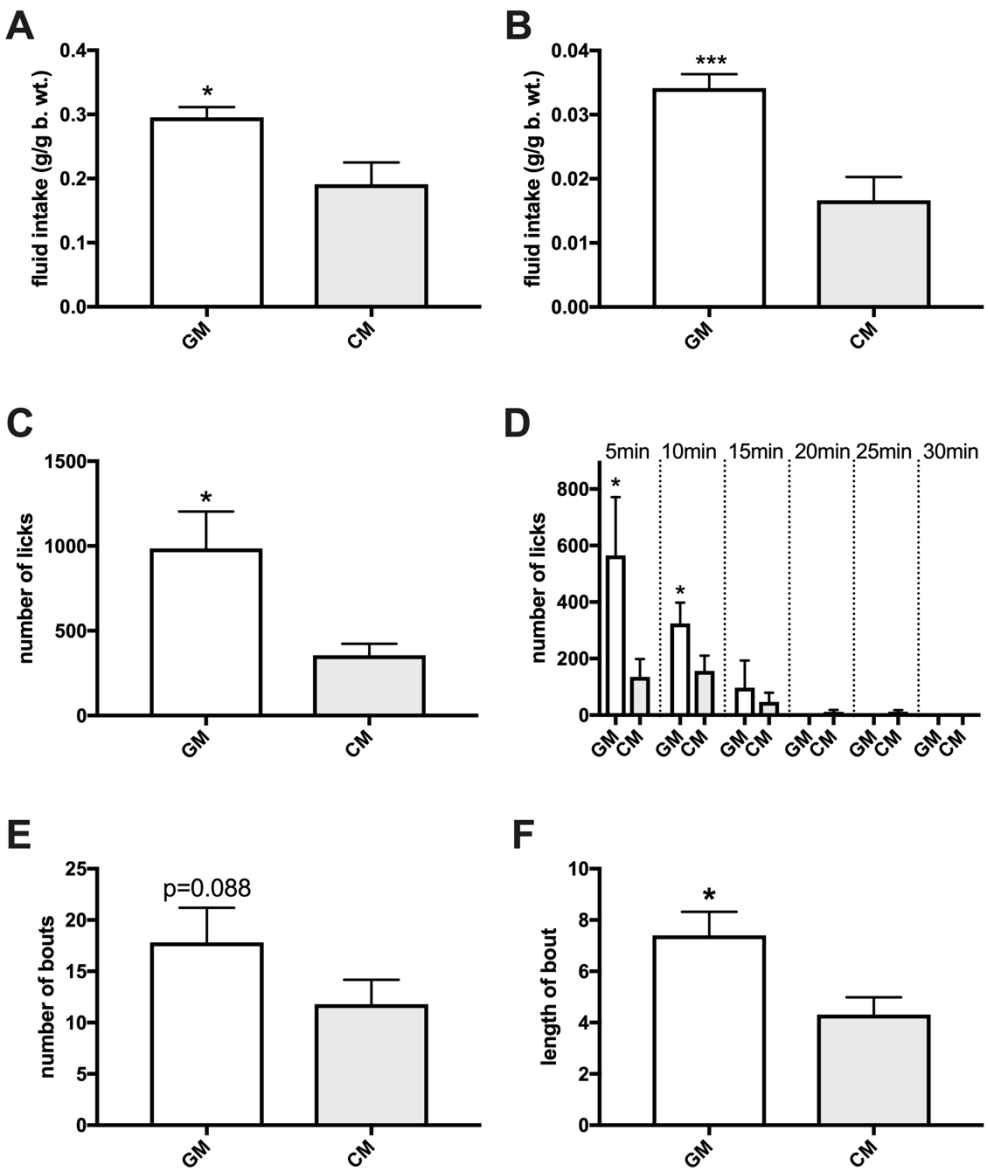

Figure 2.3: Episodic consumption of simultaneously presented GM and CM over 2-h in sated mice (**A**) and rats (**B**), lickometer activity during a 30-min exposure ((**C**): 0–30 min; (**D**): 5-min intervals), the number of GM over CM licking bouts (cluster number) (**E**), and the cluster length(s) of each GM and CM bout (**F**) in sated rats. *, $p \leq 0.05$; ***, $p \leq 0.001$.

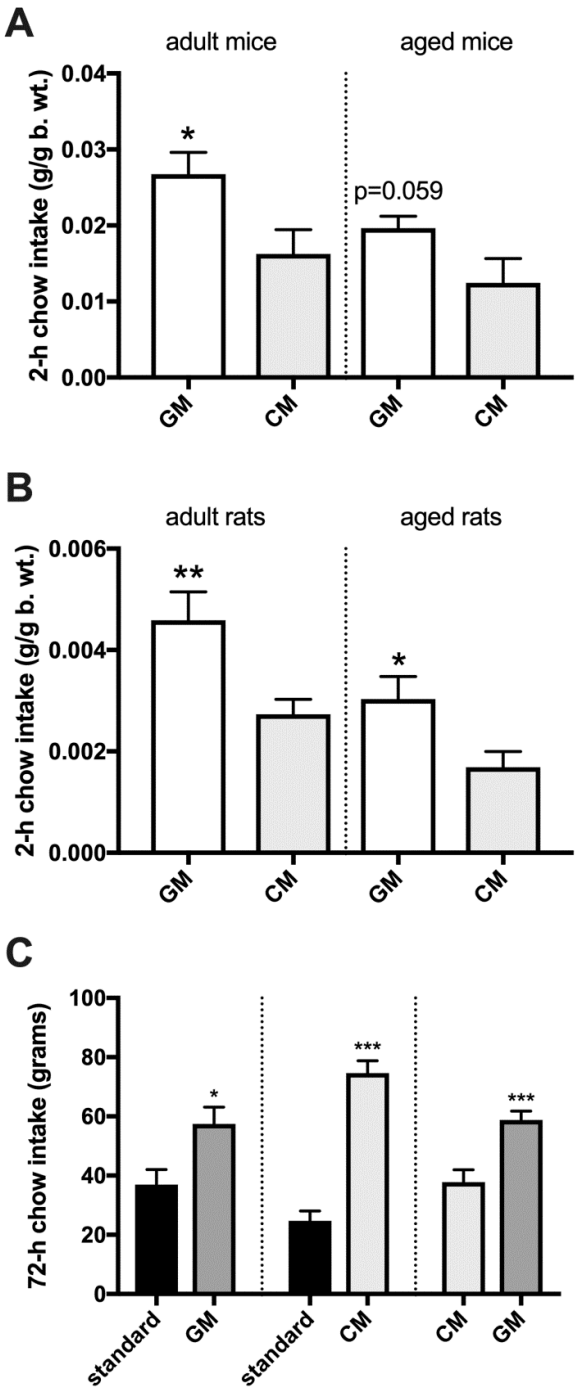

Figure 2.4 Consumption of simultaneously presented GM- and CM-enriched chow in adult and aged sated mice (A) and rats (B) over 2 h and simultaneously presented pellets (standard vs. GM; standard vs. CM, and GM vs. CM) over 72 h in adult rats (C).*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

Real-time PCR analysis after consumption of the two milk formulations (GM: 19.27 +/− 0.18 g; CM: 18.44 +/− 0.17 g) revealed that GM upregulated in the NAcc PNOC ($p = 0.0164$), ORL1 ($p = 0.0042$), Cx36 ($p = 0.0017$), GLP1R ($p = 0.0015$), MC4R ($p = 0.002$), OXT ($p < 0.001$), and GHSR ($p < 0.001$) genes, whereas mRNA levels of PENK were lower (though it did not reach significance with a p value of 0.01), compared with CM consumption. In the hypothalamus, MOR ($p = 0.045$) and KOR ($p = 0.017$) transcript levels were higher after GM consumption, and in the brain stem there was a trend toward upregulation of the MC4R ($p = 0.099$) and the MC3R was upregulated ($p = 0.0275$; Figure 2.5). Compared to water controls, in the NAcc, GM affected expression of ORL1 ($p = 0.012$), Cx36 ($p = 0.0052$), GLP1R ($p = 0.0042$), MC4R ($p = 0.0053$), OXT ($p = 0.0149$), and GHSR ($p < 0.001$); in the hypothalamus, ORX ($p = 0.0164$), KOR ($p = 0.0399$), and MC4R ($p = 0.0403$). On the other hand, hypothalamic expression of the MC4R gene was elevated by CM intake ($p = 0.041$; Figure 2.5).

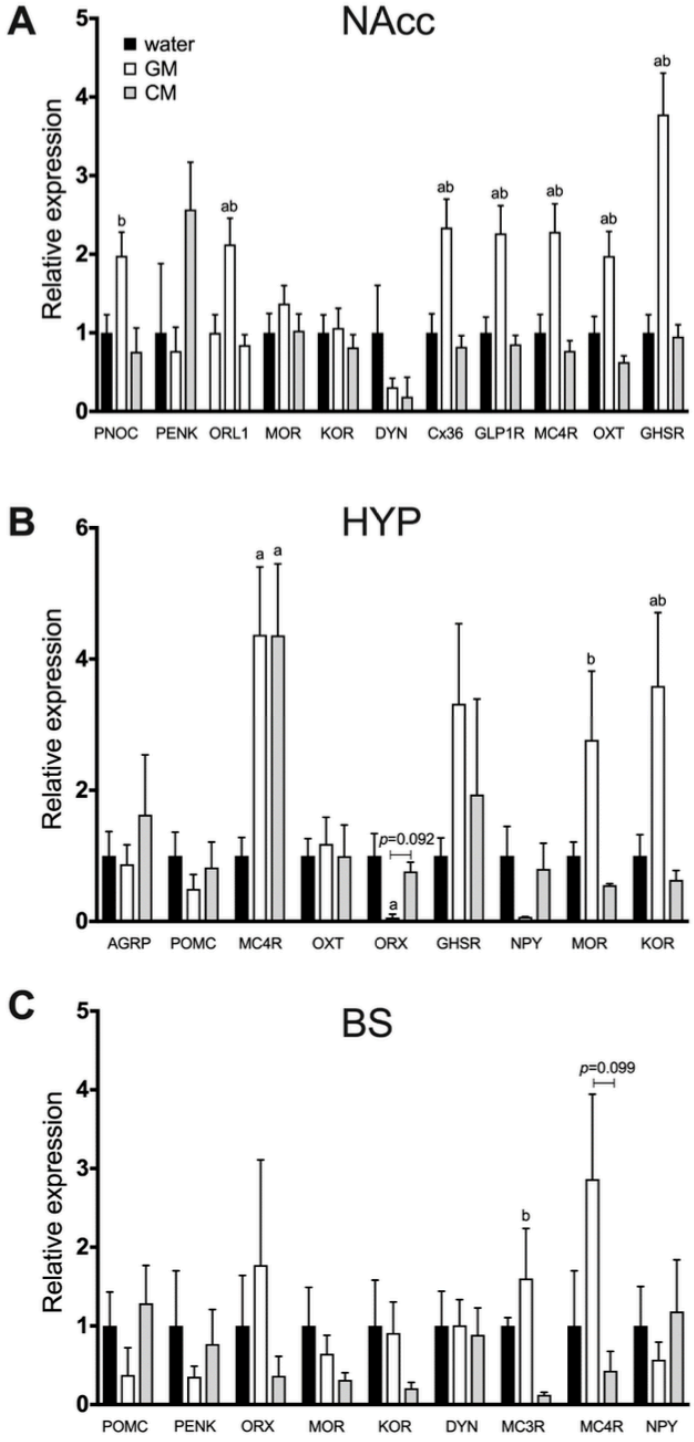

Figure 2.5: Relative expression of feeding-related genes in the nucleus accumbens (**A**), hypothalamus (**B**), and brain stem (**C**) of mice maintained for 24 h on GM or CM. Water served as a baseline tastant. [a] – significantly different from the water group; [b] – significantly different from the CM group. Analysis performed with ANOVA followed by Bonferroni's test and corrected for multiple comparisons.

2.5 Discussion

Enhanced motivation to eat in the absence of an immediate need to replenish calories or continuation of a meal beyond levels that restore energy balance typically occur when an individual is given access to food that is highly palatable. In laboratory animal models, similarly to what is observed in humans, a variety of tastants are perceived as palatable. Those include ingestants whose palatability is derived mainly from the flavour and/or postabsorptive effects of either a single macronutrient (e.g., sucrose-sweetened solutions) or from the complex contribution of multiple nutritive components (e.g., in meat rich in protein and fat) (Levine *et al.*, 2003; Martire *et al.*, 2013; Martire *et al.*, 2014). Calorie density of food (especially when coupled with high energy needs of an organism) is an additional factor that affects the liking of and preference for a given food (Drewnowski, 1998; Olszewski *et al.*, 2011).

The current set of studies show that both GM and CM and milk-enriched solid diets are highly palatable. In no-choice acceptance paradigms, energy non-deprived rats and mice of all age groups (adolescent, adult, and aged) consumed GM and CM as avidly as the calorie-matched 15% sucrose solution (used here as a positive control for a highly palatable tastant in rodents (for review, see (Levine *et al.*, 2003)), while ingesting only minimal amounts of the 'bland' cornstarch. A similar phenomenon was observed in energy-deprived animals, although the amount by which GM, CM, and sucrose intakes exceeded that of cornstarch was not as pronounced as in sated rodents. That was due to the vigorous energy deficit-driven consumption of cornstarch and a 'ceiling effect' that prevents ingestion of large amounts of the solutions during the brief refeeding period. Importantly, GM and CM enrichment of laboratory chow stimulated intake in both hungry and sated animals well above the level of standard pellets. It indicates that both GM- and CM-derived palatability is a generalised phenomenon, not limited to liquid milks, but extending to solid foods that contain milk powder. This is in concert with the ability of other palatable tastants (including, but not limited to, fat, sucrose,

and select amino acids) to have a positive gustatory effect when presented as a component of both liquid and solid foods (Moran & Ladenheim, 2016). The fact that not only adolescent and adult animals, but also the aged ones, readily consume GM and CM suggests that age-related decline in hedonic processing (Gosnell *et al.*, 1983; Landi *et al.*, 2016; Tenk *et al.*, 2017; Tomm *et al.*, 2018) does not completely abolish a drive to eat milk-based diets. Instead, a slightly depressed intake of GM and CM at an old age parallels that reported for sweet solutions, as shown here and by other authors (Shin *et al.*, 2012; Inui-Yamamoto *et al.*, 2017; Sakai *et al.*, 2017). This finding is particularly relevant from the standpoint of being able to use palatable GM or CM as nutritionally superior alternatives to, e.g., sucrose-sweetened tastants in aged individuals (Morley, 2013). That adolescent rodents also consume large quantities of both milk types indicates that prolonged dietary habituation is not required to develop the liking of either GM or CM. In fact, the amounts of GM and CM ingested by juveniles were as high as the volume of sucrose (readily consumed in large quantities by young animals, e.g., see (Naneix *et al.*, 2016)) even though the individuals had had only two brief exposures to these solutions prior to the experiment.

The single-tastant scenarios above strongly suggest a high acceptance level for both GM and CM indicating they are palatable, but as these no-choice paradigms produced fairly similar feeding responses, choice studies were needed to define relative preference for these two milk types. Simultaneous 2-h exposure to two bottles containing GM and CM showed that adult and aged mice and rats as well as adolescent rats exhibit a marked preference for GM (adolescent mice were the only cohort in which GM and CM were iso-palatable). The preference for GM did not appear to be related to whether the animals' pre-exposure to the specific diets was simultaneous (such as in adolescents and adults) or sequential (aged rodents). This finding was further expanded by employing the 30-min lickometer analysis in adult rats. It showed approximately four times as many licks at the bottle containing GM compared to CM during

the first 5 min of the meal, and twice as many licks at the GM bottle in the subsequent 5-min interval. Overall, the licking activity at both bottles occurred within the same timeframe with neither milk type being ingested in a prolonged fashion. It increases our confidence in that motivation to consume palatable GM rather than maintenance of a meal (due to, e.g., delayed satiation (Glass *et al.*, 2001)) is the main reason for avid intake of GM. The analysis of the licking bouts provides additional support for this notion. The cluster number (total number of bouts) neared significance for GM, possibly reflecting the incentive motivational properties of the food stimulus; importantly, the relationship of motivation and this measure reflects post-ingestive negative feedback (Davis & Smith, 1992; Higgs & Cooper, 1998; D'Aquila, 2010; Dwyer, 2012; Mendez *et al.*, 2015). On the other hand, the average cluster length—significantly greater for the GM formulation—typically parallels the hedonic properties (mainly, orosensory pleasure) of ingestive stimuli (as reviewed, e.g., in (Dwyer, 2012)). In this case, it is the length of clusters that appears to be the main driver for the preference for GM. A good example of the significance of licking bout length versus number in the context of neural regulation of food intake comes from studies on the endogenous opioid system. Ostlund et al. found that mu opioid receptor (MOR) knockout (KO) mice show alterations in sucrose licking: while energy-deprived wild-type mice increased burst length, relative to the nondeprived condition, this aspect of licking was insensitive to changes in food deprivation in MOR KOs. The rate of sucrose and sucralose licking in KOs was lower than in wildtype animals, providing evidence that the MOR was involved in processing palatability (Ostlund *et al.*, 2013). Mendez and colleagues reported that proenkephalin (PENK) KOs given a sucrose solution exhibited fewer bouts of licking (though the length did not differ) than wild type controls, indicating a diminished motivation to eat (Mendez *et al.*, 2015). Finally, studies on the involvement of nociceptin/orphanin FQ (NOC) revealed that NOC administration initiates new bouts of licking for sweet solutions, which is in line with the notion of its potential relationship to motivational

aspects of feeding. Interestingly, energy-deprived NOC receptor KO mice given sucrose showed longer bouts of licking than wild types, suggesting that, under hungry conditions, NOC may also affect hedonics of consumption (Mendez *et al.*, 2016).

The notion that satiety is not delayed by GM intake is supported by the experimental work exploring satiating effects of a CM- versus GM-based meal in humans. In their study, Rubio-Martín et al. presented healthy adults with GM-based or CM-based breakfast after an overnight fast and obtained blood samples and appetite ratings from the subjects just before and up to 5 h after completion of the meal. They found that that the 'desire to eat' rating was significantly lower and hunger rating tended to be lower after the GM breakfast. Interestingly, the area under the curve (AUC) for a satiety hormone glucagon-like peptide-1 was inversely associated with the AUChunger and AUCdesire-to-eat after the GM meal (Rubio-Martín *et al.*, 2017).

The aforementioned data obtained in human observations combined with the current results of our experiments in animal models suggest that even though composition differences between GM and CM are relatively minor, they are sufficient to significantly affect appetite-related parameters. It remains to be elucidated whether these effects are produced by a specific macronutrient component, a combination of nutritive components, and/or some physico-chemical characteristics of each milk type (e.g., micelle structures in GM vs. CM differ in diameter, hydration, and mineralization) (Park *et al.*, 2007).

The analysis of mRNA levels of feeding-related genes sheds more light on neural processing underlying enhanced preference for GM over CM. One of the most striking outcomes is the fact that, unlike in the NAcc, which showed an increase in multiple mRNA profiles after GM over CM, there are relatively few significant differences in gene expression in the hypothalamus and brain stem. Those two brain areas serve as the foundation for the control of energy homeostasis and consumption-related changes in the internal milieu associated with

plasma osmolality, stomach distension, and defence from exposure to food-borne toxins (Klockars *et al.*, 2019). In this network, the brain stem acts as the relay station between the periphery and the central nervous system, whereas the hypothalamus plays an endocrine role (by releasing, e.g., anorexigenic hormones, such as oxytocin (OXT) via the neurohypophysis) and innervates a number of central target sites (it includes the reciprocal connectivity with the brain stem, as well as multiple pathways with, among others, nigrostriatal and hippocampal structures). It is noteworthy that, despite the same level of intake of GM and CM over the 24-h period, the hypothalamic expression of NPY and orexin (ORX) was lower in the GM group. Both ORX and NPY in the hypothalamus enhance consumption chiefly by increasing hunger and motivating intake of energy-dense tastants (Levine *et al.*, 2004; Nixon *et al.*, 2012). Thus, these data suggest that enhanced preference for GM over CM of the shorter choice and no-choice scenarios does not stem from the stimulation of neural mechanisms that lead to hunger-driven feeding. In line with the aforementioned conclusion from feeding experiments that the increased preference for GM vs. CM in choice scenarios is unlikely to be related to suppressed satiety signalling, we found that the brain stem expression of satiation promoting melanocortin receptors (Wirth *et al.*, 2001; Girardet & Butler, 2014) is elevated after consumption of GM (it remained the same in the hypothalamus). This change in the receptor mRNA level coupled with the lack of a difference in the melanocortin ligand precursor gene expression (proopiomelanocortin, POMC) as well as in the anorexigenic OXT gene (Olszewski *et al.*, 2010; Olszewski *et al.*, 2016) suggests the lack of impairment in central satiety processing after GM (and, surprisingly, even a somewhat greater sensitivity of the molecular network promoting satiety in response to GM consumption).

Interestingly, the hypothalamic genes whose expression was elevated by GM intake were those encoding the MOR and kappa (KOR) opioid receptors (MOR and KOR brain stem and accumbal mRNA levels were also higher, though the difference did not reach statistical

significance). Furthermore, in the NAcc, we found overexpression of genes coding for opioid-related NOC and this peptide's receptor, ORL1. Opioid receptors are directly implicated in the regulation of feeding for reward (Glass *et al.*, 2001; Gosnell & Levine, 2009). They are part of a dispersed network that includes the NAcc as one of the key sites mediating hedonic aspects of eating behaviour. They are also expressed throughout the 'homeostatic' components of the feeding-related circuit (Olszewski *et al.*, 2008), including the hypothalamus and brain stem, where they are theorised to promote excessive consumption of palatable tastants by delaying meal termination. The magnitude at which opioid receptor agonists, such as butorphanol tartrate, dynorphin and beta-endorphin, stimulate consumption parallels the relative palatability of foods (Gosnell *et al.*, 1986; Gosnell & Levine, 2009). Conversely, opioid receptor antagonists, e.g., naltrexone and naloxone, are particularly effective at reducing intake of tasty ingestants (Giraudo *et al.*, 1993). Hence, higher expression of the MOR and KOR mRNA after GM is in line with the observed preference for the GM over CM. Changes in expression of additional NAcc genes that underscore the functional relationship between GM intake and reward processing include upregulation of Cx36 mRNA, as Cx36 ensures proper synchrony of dopaminergic pathways (Steffensen *et al.*, 2011), and of the growth hormone secretagogue receptor (GHSR) mRNA, considering that the GHSR in the NAcc has been found to mediate hedonics of ingestive behaviour (Skibicka *et al.*, 2013). Again, as in the case of the hypothalamic gene expression analysis, genes encoding molecules that promote satiety – such as OXT, melanocortin receptor 4, and glucagon-like peptide-1 receptor (Kanoski *et al.*, 2016) – were upregulated after GM, which points to the heightened reward processing rather than impaired satiation as the factor propelling preference toward GM over CM.

2.6 Conclusions

In laboratory animal models, GM and CM are highly palatable when presented as liquids and as components of solid diets. Diet choice paradigms reveal preference for GM over CM in mice

and rats belonging to different age groups. Feeding studies and analyses of gene expression in the feeding-relevant brain circuit point to feeding reward as the main factor underlying preference for GM. The complex nutritional profile of milk varies between species. With the use of skim milk with low lipid content, macronutrient variation eliciting preference for GM over CM would be attributed to either lactose or milk protein fractions. Incorporation of milk into human diets comes with modification of milk composition, often with adjustment of the protein fraction. The predominance of CM in western societies is reflected in a bias in literature concerning acceptance and postprandial effects of whey and casein fractions, the two major milk proteins, in which CM proteins are widely utilised over other species milks. There is suggestion that modifying protein fractions in milk diets alters digestive and post-absorptive consequences. However, with differences in protein compliments between CM and GM and the variation in acceptance and preference observed here, little is known regarding acceptance of modified whey and casein content across species milks with altered patterns of intake, digestion, endocrine and central responses.

2.7 References

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Chapter 3

Changes in feeding and related brain activity and gene expression in mouse model following consumption of goat's milk formulations with variable whey and casein content.

3.1 Abstract

In the previous chapter, preference for goat's milk (GM) over cow's milk (CM) was shown in laboratory animal models in choice scenarios. Across different ages, animals exhibited avid consumption of GM-based liquid and solid diets. Preference was driven with reward-related signalling in opioidergic and dopaminergic systems. In this chapter, I describe the modifications to feeding behaviours and central signalling following GM-derived formulations with natural and adjusted whey to casein content. Adjustment of protein content in animal milk-based formulations is done to modify protein and energy levels, to ensure adequate amino acid intake and to affect satiety. The shift from the natural whey:casein ratio of 20:80 in animal milk formulations for adults and for infants is oftentimes done to reflect the 60:40 whey:casein ratio of human milk. Recent studies show altered digestion and metabolic parameters accompany modified whey:casein – *in vitro* and adult studies showed that 20:80 versus 60:40 whey:casein milks differently affect casein proteolysis rate, glucose metabolism and hormone release; these data are supported by animal model findings. Importantly, it is unknown whether the adjustment from the 20:80 to 60:40 ratio affects appetite and brain processes related to food intake. The current set of studies therefore focused on the impact of the 20:80-to-60:40 whey:casein content shift in GM-derived formulation on food intake and feeding-related brain processes in the adult organism. In laboratory mice the 20:80 whey:casein GM formulation was consumed less avidly and was less preferred than the 60:40 formulation in short-term

choice and no-choice feeding paradigms. Appetite changes were reflected by altered hindbrain and hypothalamic mRNA expression of genes relevant to feeding, including the melanocortin system. They were also associated with changes in brain neuronal activation patterns assessed by the analysis of an immediate early gene product, c-Fos, in the nucleus of the solitary tract, area postrema, ventromedial hypothalamic nucleus and supraoptic nucleus. It is concluded that the shift from the 20:80 to 60:40 whey:casein ratio in GM-derived formulations affects short-term feeding and relevant brain processes.

3.2 Introduction

In Chapter 2, variation in intake of goat's milk (GM) and cow's milk (CM) was described in laboratory animals. Rat and mice models exhibited avid consumption of GM over CM in both liquid and solid diets and across a range of ages. Analysis of relative gene expression in key brain regions regulating feeding noted altered expression of reward-related genes in opioidergic, dopaminergic and oxytocin-related pathways. Elevated consumption of GM driven by reward reinforces species difference in consummatory outcomes, previously described in variable digestion and peripheral post-absorptive nutrient and hormone status. Chapter 2 serves as the first description of modified acceptance and preference with GM and CM, expanded upon in the next two chapters examining impact of modifying protein content of milk on appetitive behaviours.

Milk and dairy products constitute a significant proportion of a typical diet and the nutritional benefits associated with their consumption stem from, among others, the macronutrient profile of milk, including the unique protein composition (Anderson & Moore, 2004; Chen *et al.*, 2014; Hirahatake *et al.*, 2014; Pasiakos, 2015). Milk proteins consist primarily of whey and casein (Jahan-Mihan *et al.*, 2011). Unlike the 60:40 whey:casein ratio of human milk, the protein fraction of animal milks (such as bovine and caprine milk predominantly available on

the consumer market) has the natural whey:casein ratio of approximately 20:80 (Park & Haenlein, 2013); and thus milk formulations used in human nutrition – most commonly infant formulas – are often whey-enhanced to match the 60:40 ratio (Goedhart & Bindels, 1994; Lönnerdal, 2003; Heird, 2007).

It is well established that proteins, including those present in milk, affect appetite, body weight and metabolic parameters (Anderson & Moore, 2004; Zemel, 2004; Jahan-Mihan *et al.*, 2011). Importantly, data show that whey and casein generate distinct physiological and appetitive responses by interacting with specific transporters and receptors in the gut, affecting nutrient absorption, modifying gastric emptying and gastrointestinal (GI) hormone release (Boirie *et al.*, 1997b; Dangin *et al.*, 2003; Anderson & Moore, 2004; Jahan-Mihan *et al.*, 2011). Whey and casein have unique digestion kinetics and post-absorptive effects. Digestion of whey is rapid compared to casein: casein proteins aggregate into curds (Luiking *et al.*, 2016; Wang *et al.*, 2018), delaying delivery of constituent metabolites to the intestine (Boirie *et al.*, 1997b; Marsset-Baglieri *et al.*, 2014; Meyer *et al.*, 2015; Dalziel *et al.*, 2017). Plasma amino acid levels reflect digestion speed, with whey intake inducing higher, immediate increases in circulating amino acids (Boirie *et al.*, 1997a; Hall *et al.*, 2003; Calbet & Holst, 2004) and casein having delayed and lower but sustained hyperaminoacidemia (Boirie *et al.*, 1997a).

Consequently, whey and casein differentially influence the release of some consumption-regulating hormones, which – in turn - likely produces a unique downstream central nervous system response, including activity of relevant brain systems that control appetite. While both fractions produce hypophagia via peptide YY (PYY) and its interaction with the Y2 receptor (Reidelberger *et al.*, 2013; Zapata *et al.*, 2018), whey is a much more potent enhancer of cholecystokinin (CCK), glucagon-like protein 1 (GLP-1) and glucose dependent insulinotropic polypeptide (GIP) release (Hall *et al.*, 2003; Marsset-Baglieri *et al.*, 2014; Hoefle *et al.*, 2015;

El Khoury *et al.*, 2019). As plasma amino acids and gastrointestinal hormones influence feeding either through direct or vagal-mediated action on central pathways (Gietzen *et al.*, 1989; Blouet *et al.*, 2009; Gartner *et al.*, 2018a; Gartner *et al.*, 2018b; Heeley *et al.*, 2018), scarce prior literature indeed suggests differing effects of whey and casein at central feeding-related circuits, with e.g. whey more effectively modulating serotoninergic activity (Orosco *et al.*, 2004; Choi *et al.*, 2009) and expression of select energy homeostasis regulatory genes (Potier *et al.*, 2009; Andreoli *et al.*, 2016; Nilaweera *et al.*, 2017). Altered feeding patterns resulting from consumption of either fraction alone have been reported (Hall *et al.*, 2003; Marsset-Baglieri *et al.*, 2014; Pal *et al.*, 2014; Singh *et al.*, 2016).

While evidence delineating the physiological responses to individually presented whey and casein exists, human or laboratory animal studies evaluating the physiological impact of those fractions ingested in milk formulations in two commonly encountered ratios, i.e., whey:casein 60:40 and 20:80, are very scarce. This gap in knowledge is particularly surprising considering that these are common ratios in both adult and infant dairy-based nutrition, and one should not simplistically assume that the effect of combined whey and casein in milk formulation would be either negligible or merely 'proportional' to their adjusted content. In their 2019 study, (El Khoury *et al.*, 2019) found that in healthy adults given a 60:40 versus 20:80 whey:casein milk beverage, a higher whey:casein ratio milk ingested along with high-carbohydrate cereal decreased postprandial glycemia in an insulin-independent manner, primarily through delayed gastric emptying (El Khoury *et al.*, 2019). The authors also observed the preprandial glucose peaks to be lower and GLP-1 plasma levels to be elevated after ingestion of milk with the 60:40 ratio. In line with that, obese rats showed greater improvements in glucose tolerance when fed whey than those given whey plus casein (Nilsson *et al.*, 2007; Pezeshki *et al.*, 2015).

Even though the search for an improved whey:casein ratio has been largely spurred by the intent to improve eating behavioural, nutritional and metabolic consequences of protein consumption, surprisingly, very little is known about potential appetite-related feeding and neural consequences that the departure from the natural 20:40 toward 60:40 whey:casein protein ratio in animal milk formulation may produce. This gap in knowledge is particularly critical since it can be presumed that the distinct feeding and neuroendocrine effects shown for whey and casein alone likely contribute to unique appetite-related changes induced by consumption of a formulation containing whey:casein combination at the ratio of 20:80 versus (whey-enhanced) 60:40. Surprisingly, the potential effects of such modification have never been studied. Therefore, the current study utilising adult laboratory mice was designed to determine whether an adjustment of the whey:casein ratio in protein-matched milk formulation from 20:80 to 60:40 (a) affects palatability and acceptability of the milk formulation in short-term feeding paradigms, (b) whether it evokes a different pattern of activity in feeding-related brain sites after ingestion of the matched amount of one of the formulas, and (c) whether it promotes changes in expression of hypothalamic and brainstem genes critical in food intake regulation. Given the fractions' differences in post-ingestive effects, it was speculated that appetite changes come through central mechanisms uniquely affected by modifications in whey-to-casein content. A standard caprine milk-based formula with the 20:80 versus 60:40 whey:casein ratio was used in the studies.

3.3 Material and Methods

3.3.1 Animals

Adult male C57Bl mice were single-housed in a temperature-controlled (22 °C) room with a 12:12 hour LD cycle (lights on 0900). Animals had ad libitum access to standard chow (Diet 86, Sharpes Stock Feed, Wairarapa, New Zealand) and tap water unless stated otherwise. Groups were weight-matched. The procedures were approved by the University of Waikato animal ethics committee (approval #1057).

3.3.2 Milk formulations

The formulations were GM-based (Dairy Goat Cooperative, Hamilton, New Zealand). The control GM formula contained the natural protein ratio of 20% whey and 80% casein (20:80 GM) whereas the novel formula had 60% whey and 40% casein (60:40 GM). They were stored as powder and prepared immediately before use by being reconstituted in water. All animals were pre-exposed to the formulas prior to the trials in order to prevent neophobia. Composition of formulations are detailed in Table 3.1.

Table 3.1: Nutritional composition of GM formulations per 100 ml

	kJ	Protein (g)	Whey protein (%)	Fat (g)	Carbohydrate (g)
20:80 GM	278.1	1.3	20.0	3.5	7.5
60:40 GM	275.5	1.4	60.0	3.5	7.1

3.3.3 Feeding studies

GM-based formulation treatment paradigms for adult mice are outlined in Table 3.2

Table 3.2: Schematic of GM-based formulation treatment paradigms in adult mice

	 Sated	 Deprived	 Adult	 Mouse
Single tastant				
GM formulations - 1 hour	X		X	X
GM formulations - 2 hour		X (with chow)	X	X
GM formulations - 24 hour	X		X	X
Choice				
GM formulations 2 bottle choice	X		X	X

3.3.3.1 Preference for the simultaneously presented GM formulas

Mice (n=7-8/group) were acclimatised to two-bottle presentation of the formulations on two separate occasions one week prior to the trial. On the experimental day at 10:00, chow and water were removed from the cages and mice were simultaneously given access to two bottles, one containing the 20:80 GM formulation and the other, the 60:40 GM solution. Intake was measured after 2 hours by weighing the bottles and the data were expressed in grams.

3.3.3.2 Energy deprivation-induced 2-hour intake of each GM formulation presented individually along with standard chow

Mice (n=10/group) were deprived overnight of chow; water was available during that time. At 10:00 they were given access to standard chow and a bottle containing either the 20:80 GM formulation or the 60:40 GM solution for 2 hours. Water was removed during the 2-hour meal as the formulas were the source of both calories and water. In an additional control scenario, in order to determine the impact of the formulations on consumption of standard chow, another group of mice (n=10) was refed with chow, but instead of either formulation, they received a bottle of water. Chow and fluid consumption was determined at the end of the 2-hour meal.

3.3.3.3 Intake of the formulas presented independently for 24 hours

During a 24-hour pre-exposure period, to reduce neophobia, a water bottle in each cage was replaced with a bottle containing either the 20:80 GM formulation or the 60:40 GM test solution (chow was available). On the experimental day, both chow and water were removed (start at 09:00) and a bottle containing either the 20:80 or the 60:40 GM formulation was placed in the cage. The formulations were the only source of both calories and fluid for the next 24 hours. Afterwards, formulation intake was measured in grams.

3.3.4 Neuronal activation in feeding-related hypothalamic and brainstem areas after consumption of the same amount of the 60:40 GM versus 20:80 GM formulation.

Protooncogene c-fos is an immediate gene product elevated during activation of the neurone, with peak expression 30-60 minutes post-stimuli (Morgan & Curran, 1991), allowing tracing of central activity following food intake. The purpose of this experiment was to assess whether consumption of the same amount of the 60:40 GM formula induces a different pattern of neuronal activation in areas of the hypothalamus and brain stem that are crucial in the regulation of food intake compared to the 20:80 GM formula.

Water and standard chow were removed from the cages and animals were presented with either 20:80 or the 60:40 GM formulation (n=8) for 1 hour and allowed to drink ~1 g/g BW . Mice were then anaesthetised with intraperitoneal 35% urethane and perfused with saline (10 ml) followed by 50 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) one hour after termination of diet exposure. Brains were dissected and postfixed in PFA at 4 °C overnight. Coronal 60 μm vibratome (Leica, Germany) sections were processed for c-Fos immunostaining. The sections were incubated in 3% H_2O_2 in 10% methanol (in tris-buffered

saline (TBS); pH 7.4) for ten minutes, then overnight in rabbit anti-c-Fos antibody (1:3000; Synaptic Systems, Goettingen, Germany) at 4 °C. Then sections were incubated for 1 hour in the secondary biotinylated goat-anti-rabbit antibody (1:400; Vector Laboratories, USA) and for 1 hour in avidin-biotin complex (1:800; Vector Laboratories, USA) at room temperature. 0.05% DAB, 0.01% H_2O_2 and 0.2% nickel sulphate (Sigma, USA) were used to visualise cFos-positive nuclei. All incubation utilised a mixture of 0.25% gelatin and 0.5% Triton X-100 (Sigma, USA) in TBS. TBS was also used for intermediate rinsing. Sections were mounted onto gelatinised slides, dried and dehydrated in ascending concentrations of ethanol, soaked in xylene and embedded in and embedded in Entellan (Merck, Germany). Manual counting of c-Fos immunoreactive nuclei was performed bilaterally in all regions (4-5 sections/animal) by a person blinded to group allocations at 10x and 40x magnifactions on Nikon microscope. Densities of c-Fos positive nuclei/mm^2 were averaged per group.

3.3.5 Hypothalamic and brainstem gene expression following 24-hour exposure to the 20:80 GM vs 60:40 GM formula

Upon completion of the 24 hour 20:80 or 60:40 GM formula exposure (as described in Section 3.3.3.3), the animals were sacrificed at 09:00 by cervical dislocation and the brain stem and hypothalamus were dissected. They were stored in 1 ml RNALater (Invitrogen, USA) at room temperature for 1 hour and then at -80°C until processing.

Upon thawing, the tissue was transferred from RNALater to TRIzol (Life Technologies, USA; 1ml/100mg tissue) and mechanically homogenised. Chloroform (0.2ml/100mg tissue) was added and samples were centrifuged at 4 °C for 20 minutes at 10,000× *g.* The clear phase containing RNA was siphoned, 0.5ml isopropanol was added and samples were put on ice for 10 minutes. Samples were centrifuged again at 4 °C for 20 minutes at 10,000× *g*. The aqueous

phase was removed from the pellets which were resuspended in 0.3ml ethanol and centrifuged at 4°C for 10 minutes at 10,000× *g*. Ethanol was removed and the pellets were air-dried.

8 µL of DEPC H_2O and 1 µl of DNAse buffer (dNature, New Zealand) was added to the pellets. These were incubated with 1 µl DNAse (dNature, New Zealand) at 37 °C for 30 minutes. DNAse was inactivated with 1 µl stop buffer (dNature, New Zealand) addition and incubation at 67°C for 10 minutes. Removal of genomic DNA was confirmed via PCR using HOT FIREPol Blend Master Mix (dNature, New Zealand), then agarose gel electrophoresis. RNA concentrations were measured with a nanodrop.

cDNA was synthesised from RNA using iScript Advanced cDNA synthesis kit (BioRad, New Zealand), confirmed with PCR followed by agarose gel electrophoresis. RT-qPCR determined relative expression levels of housekeeping genes (ActB, β-tubulin, H3B) and genes of interest. Reactions contained 4 µl of 25 ng/µl sample cDNA, 1 µl of each forward and reverse primers (5 µM), 10 µl iTaq Universal SYBR Green Supermix (BioRad, New Zealand) and 4 µl MilliQ water. Reactions were performed in duplicates alongside MilliQ water negative controls for each primer pair. Amplification protocol was initiated at 95 °C for 15 minutes, followed by 45 cycles of 15 seconds at 95°C, 15 seconds at the primer-specific annealing temperature and 30 seconds at 72 °C. Primers used are detailed in Table 3.3.

Table 3.3: Forward and reverse primers for housekeeping and target genes used in RT-qPCR analyses of hypothalamic and brainstem relative gene expression following GM formulations

Gene	Forward	Reverse
ACTB	5′-AGTGTGACGTTGACATCCGT-3′	5′-TGCTAGGAGCCAGAGCAGTA-3′
BTUB	5′-CGGAAGGAGGCGGAGAGC-3′	5′-AGGGTGCCCATGCCAGAGC-3′
H3B	5′-CCTTGTGGGTCTGTTTGA-3′	5′-CAGTTGGATGTCCTTGGG-3′
MC4R	5′-CTTATGATGATCCCAACCCG-3′	5′-GTAGCTCCTTGCTTGCATCC-3′

POMC	5′-GACACTGGCTGCTCTCCAG-3′	5′-AGCAGCCTCCCGAGACA-3′
NPY	5′-GGTCTTCAAGCCGAGTTCTG-3′	5′-AACCTCATCACCAGGCAGAG-3′
KOR	5′-CACCTTGCTGATCCCAAAC-3′	5′-TTCCCAAGTCACCGTCAG-3′
MOR	5′-CCTGCCGCTCTTCTCTGG-3′	5′-CGGACTCGGTAGGCTGTAAC-3′
DYN	5′-GACAGGAGAGGAAGCAGA-3′	5′-AGCAGCACACAAGTCACC-3′
OXT	5′-CCTACAGCGGATCTCAGACTG-3′	5′-TCAGAGCCAGTAAGCCAAGCA-3′
ORX	5′-GCCGTCTCTACGAACTGTTGC-3′	5′-CGCTTTCCCAGAGTCAGGATA-3′
PNOC	5′-AGCACCTGAAGAGAATGCCG-3′	5′-CATCTCGCACTTGCACCAAG-3′
OPRL1	5′-ATGACTAGGCGTGGACCTGC-3′	5′-GATGGGCTCTGTGGACTGACA-3′

3.3.6 Statistical analyses

Food intake and immunohistochemistry data were analysed with unpaired student's *t*-test for two-group comparisons. In the case of the feeding study where three groups were compared with each other, a one-way ANOVA followed by Tukey's post-hoc test with a correction for multiple comparisons was used. Analyses of qPCR data were performed with BioRad CX Manager software (BioRad, New Zealand), followed by unpaired student's *t* test. Differences were considered statistically significant at $p < 0.05$.

3.4 Results

During a 2-hour two-bottle test in which the animals had a choice between the 20:80 and 60:40 GM formulations, mice showed a significantly lower preference for the 20:80 formulation (Figure 3.1, $p<0.0001$), When the formulations were given independently (no choice between the formulas) along with the standard chow for 2 hours to overnight-deprived mice, the animals that had access to the 60:40 solution drank more than the mice given the 20:80 formula (Figure 3.2, $p=0.019$). Chow intake did not differ between the two groups. Importantly, the comparison with the group that received water instead of a formula revealed that both formulas were preferred over water ($F(2,27)=15.40$; water vs 20:80 GM – $p=0.034$; water vs 60:40 GM –

p<0.001). Chow intake was lower in the formula groups than in water-given mice showing a strong trend approaching significance (water vs 20:80 GM – p=0.058; water vs 60:40 GM – p=0.065). Finally, in the 1-hour and 24-hour no-choice exposure to the 20:80 versus 60:40 GM formulations, mice drank equal volumes during the 1-hour exposure and less of the 20:80 GM solution in 24-hour exposure (Figure 3.3, P<0.001).

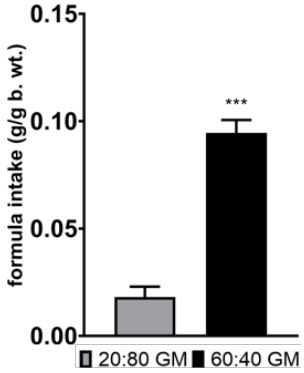

Figure 3.1 Non-deprived animals consume less of the 20:80 whey:casein formulation than of the 60:40 GM formula during a 2-h episodic exposure of simultaneously presented diets. *** $P \leq 0.001$.

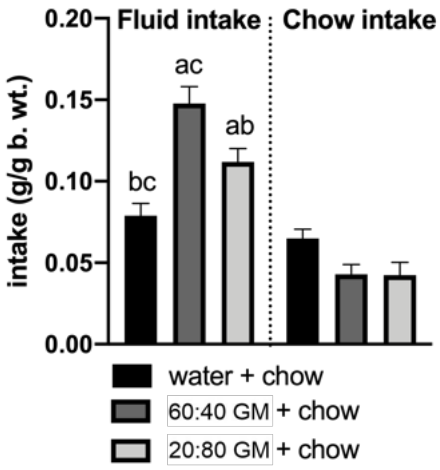

Figure 3.2: Mice overnight-deprived of food and refed for 2 h with 20:80 whey:casein GM formula + chow or with the 60:40 whey:casein GM formulation + chow or with water + chow most avidly ingested the 60:40 formula followed by the 20:80 GM solution and water. a –

significantly different from water intake; b – significantly different from 60:40 GM formula; c – significantly different the 20:80 GM formula intake. Significant when $p<0.05$.

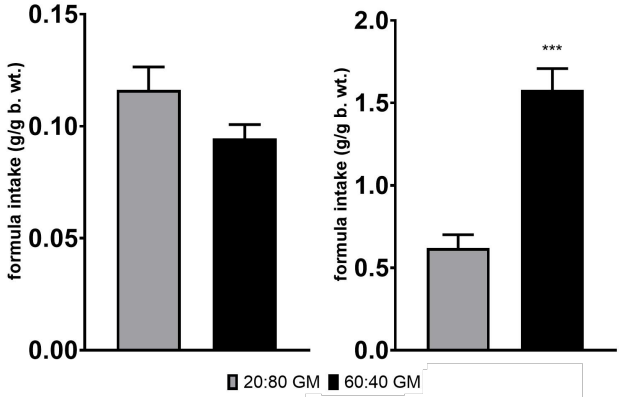

Figure 3.3: Mice given for 1 hour (left) or 24 hours (right) a single bottle of 20:80 or adjusted 60:40 GM formulations avidly consumed the 60:40 formula during the 24 hour period. *** - $P \leq 0.001$.

A decrease in cFos immunoreactivity was observed in the hypothalamic supraoptic nucleus (SON; P=0.025) as well as the ventromedial hypothalamus (VMH; P=0.008) and the rostral nucleus of the solitary tract (rNTS; P=0.0308) after 1-hour exposure to the 20:80 whey:casein GM formula compared to the 60:40 diet (Figure 3.4). Increases in cFos IR were noted in the area postrema (AP, P=0.0066) and the caudal portion of the nucleus of the solitary tract (cNTS; P=0.0165).

Real-time PCR analyses showed an increase in brainstem relative expression of the melanocortin receptor 3 (MC3R; p=0.03), orexin (ORX; p=0.028), oxytocin (OXT; p=0.003) and pro-opiomelanocortin (POMC; p=0.014) genes following consumption of the 20:80 GM formulation compared to the 60:40 formula (Figure 3.5). Increased expression of glucagon-like peptide-1 receptor (GLP-1R; p=0.033) and ORX (p=0.027) in the hypothalamus was also found with exposure to the 20:80 GM 20:80 formulation.

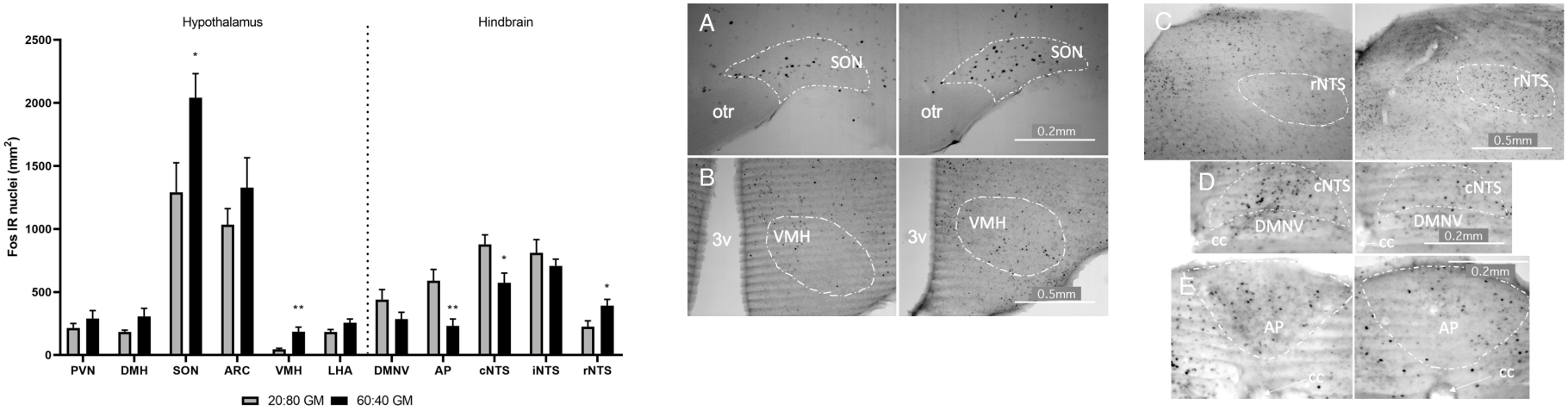

Figure 3.4: c-Fos immunoreactivity in brain sites related energy homeostasis increased in the supraoptic nucleus (SON), ventromedial hypothalamus (VMH) and rostral nucleus of the solitary tract (rNTS) and decreased in the area postrema (AP) and caudal nucleus of the solitary tract (cNTS) following the intake of the 60:40 versus 20:80 whey:casein GM formulation in mice that ingested equal volume of fluid during a 1-hour session. Photomicrographs depict c-Fos in hypothalamic (A, B) and hindbrain (C-E) areas with significant change was noticed (20:80 GM formula: left panels; 60:40 formula: right panels). PVN – paraventricular nucleus; DMH – dorsomedial hypothalamic nucleus; ARC - arcuate nucleus; LHA – lateral hypothalamic area; DMV - dorsal motor nucleus of the vagus; iNTS – intermediate nucleus of the solitary tract; otr – optic tract; 3v – third ventricle; cc – central canal; * - $P \leq 0.05$; ** - $P \leq 0.01$.

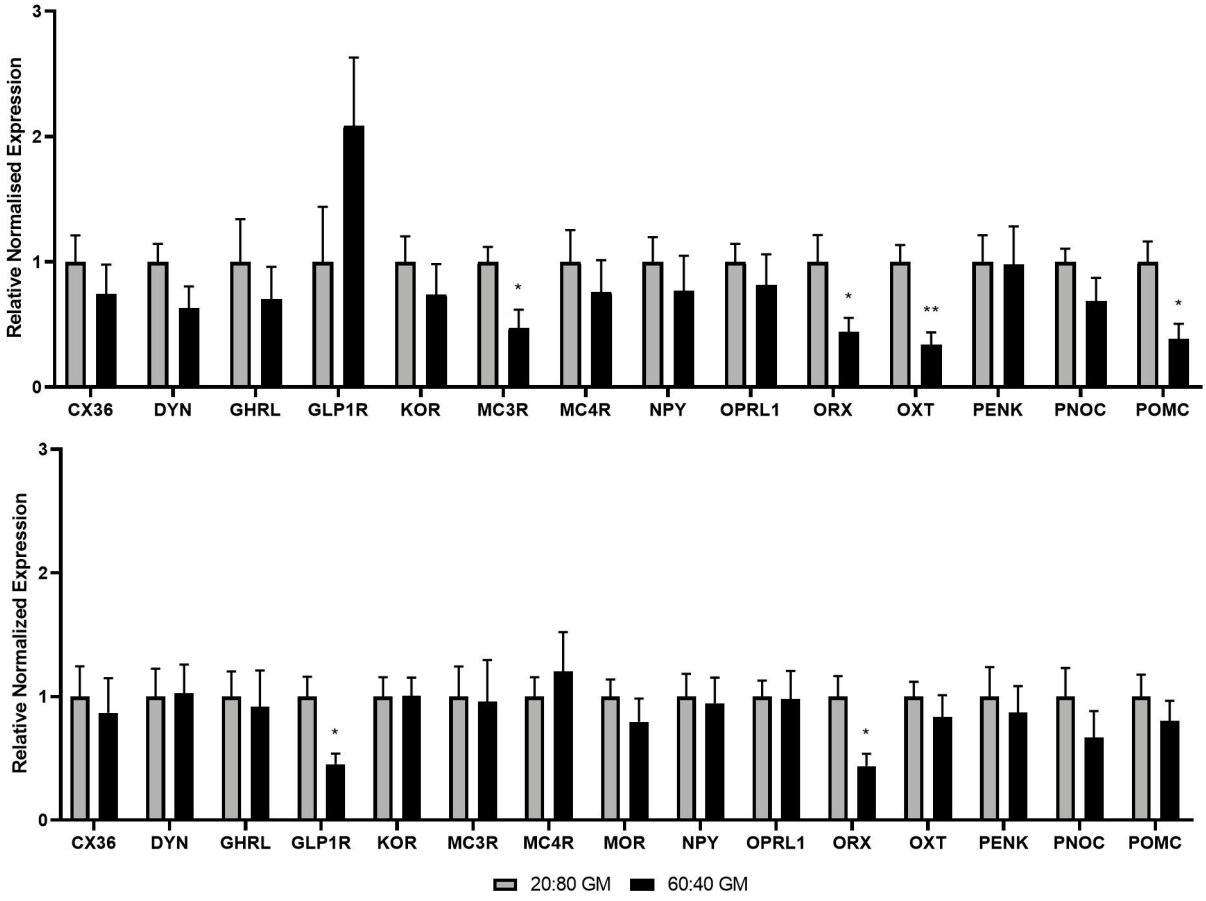

Figure 3.5: Relative expression of feeding-related genes in the brain stem (top) and hypothalamus (bottom) after 24-hour consumption of the 20:80 whey:casein GM formulation versus the 60:40 whey:casein test solution. Lower brainstem expression of melanocortin receptor 3 (MC3R), orexin (ORX), oxytocin (OXT) and pro-opiomelanocortin (POMC) was observed after the 60:40 formulation consumption. Lower expression of glucagon-like protein 1 receptor (GLP-1R) and ORX was noted in the hypothalamus after exposure to this diet. * - $P \leq 0.05$; ** - $P \leq 0.01$.

3.5 Discussion

While adequate protein intake ensures availability of amino acids, especially the essential ones, and thus supports basic functioning of the organism, excessive protein load may promote adverse effects, such as acidosis or hypercalciuria potentially resulting in kidney disease. It is not surprising, therefore, that intake of protein is a highly regulated process. On the one hand, hunger increases a drive to seek all macronutrients, including protein. However, ingestion of high-protein food triggers early termination of consumption by promoting rapid satiation; and diets very high in protein are perceived as less palatable and their acceptability is relatively low. Although the phenomenon of protein intake control has been well described in human and laboratory animal studies, surprisingly little is known about the impact of modifications in protein fraction ratios on appetite. This lack of information is particularly critical in the context of adjusting whey:casein ratios from 20:80 content (i.e., closely resembling animal milks predominant on the consumer market, such as bovine and caprine) to 60:40 in milk formulations.

The current study shows for the first time that a shift from the 20:80 to 60:40 whey:casein ratio in a formulation affects short-term consumption. Also intake of the 60:40 whey:casein milk produces a different neuronal activation pattern in feeding-related brain areas and a different expression of genes regulating food consumption in the hypothalamus and brain stem than does the 20:80 whey:casein standard formulation.

Notably, in both the 2-hour and 24-hour exposure paradigms, regardless of the presence of other tastants, the 60:40 whey:casein formulation was consumed in larger quantities and it was preferred over the 20:80 ratio. This consistent outcome across the paradigms employed in this basic research project serves as compelling evidence in that a shift from the 20:80 to 60:40 whey:casein ratio influences acceptability of and preference for milk formula in the short-term and it may potentially translate to long-term consequences for energy homeostasis. Structural

differences between whey and casein and, thus, disparate digestive and postabsorptive responses they evoke, to some extent explain how a change in the proportion of these two fractions contributes to feeding. Casein micelles coalesce in the stomach and form a curd, whereas whey passes through the stomach intact (Luiking *et al.*, 2016; Wang *et al.*, 2018). The relative speed of whey digestion is reflected in absorptive processes where more rapid availability of amino acids increases rate of uptake. Whey produces rapid transient peaks in plasma amino acid content whereas delayed gastric emptying of caseins produces a slower but prolonged elevation of amino acids (Boirie *et al.*, 1997a; Hall *et al.*, 2003; Calbet & Holst, 2004). Whey-enhanced formulations are more susceptible to heat-induced protein glycation (Meyer *et al.*, 2011; Prosser *et al.*, 2019) that may reduce their digestibility (Wada & Lönnerdal, 2015) and perturb the gut bacteria (Seiquer *et al.*, 2014). Not surprisingly, anorexigenic hormone levels vary upon whey versus casein consumption (Blouet *et al.*, 2009; Potier *et al.*, 2009; Pal *et al.*, 2014; Meyer *et al.*, 2015; Andreoli *et al.*, 2016; Singh *et al.*, 2016). For example, addition of whey to diets fed to obese rats increases PYY mRNA expression and secretion as well as feeding; and the reduction in food intake is reversed by PYY receptor-2 antagonists. Changes in brain activity have been noted in serotonergic and energy regulating pathways (Semon *et al.*, 1987; Travers, 2002; Seiquer *et al.*, 2014; Stratford *et al.*, 2017; Jinno *et al.*, 2020), though the latter ensues after long-term feeding.

In the context of the general understanding of whey and casein influence on feeding-related mechanisms, the data obtained here further support the notion that while each of the protein fractions alone specifically alters appetite and appetite-related physiological parameters, actual effects of the combined fractions cannot be simplistically extrapolated as proportional to the mere whey:casein ratio. In fact, generalization of appetitive and metabolic effects of whey and casein may be far from possible unless studied in conjunction with specific ratios and with specific foods in which these fractions are used. Indeed, data obtained in previous reports in which only one fraction or the other was added to diets or administered as a preload, are

confusing and oftentimes contradictory. For example, some authors suggested that whey might be suppressing food intake more effectively than casein (Hall *et al.*, 2003; Pal *et al.*, 2014). On the other hand, Marsset-Baglieri *et al.* (2014) found that a liquid snack of whey or casein alone or in combination was effective in suppressing appetite in overweight subjects compared to a maltodextrin control snack, however, there was no difference in satiation potency between the protein groups (Marsset-Baglieri *et al.*, 2014). Potier *et al.* (2009) gave adult subjects a cheesy snack containing either casein or whey+casein (66:33) as a meal preload (Potier *et al.*, 2009). While the preloads lowered intake at the subsequent meal, no differences were observed between the casein and whey+casein groups.

In order to identify the feeding-related physiological consequences of the departure from the conventional 20:80 to the 'whey-enhanced' 60:40 ratio, activation and neuronal activation changes were examined in the hypothalamic and hindbrain circuits relevant to appetite regulation. In the brainstem, c-Fos immunoreactivity was increased in the rostral nucleus of the solitary tract (rNTS) and decreased in the area postrema (AP) and caudal nucleus of the solitary tract (cNTS). Immediate response to the 60:40 whey:casein content appears to incorporate gustatory-related signalling through increased rNTS activation, a region with significant gustatory and sensory input (Rinaman, 2010). The rNTS displays enhanced activity following oral delivery of strong flavoured tastants, such as sweet sucrose, bitter quinine, or sour citric acid (Harrer & Travers, 1996; King *et al.*, 1999; Travers, 2002; Stratford *et al.*, 2017). Additionally, activity in the cNTS suggests a role of visceral input contributing to appetitive behaviours. Vagal efferents terminating in the cNTS and the relative permeability of the blood brain barrier in the brain stem allow the combined visceral sensation and circulating nutrients to modulate activity of broader brain pathways (Horst *et al.*, 1989; Rinaman, 2010). The NTS projects extensively to energy homeostasis-related and appetite regulating regions including the PVN, LHA and DMH (Horst *et al.*, 1989).

In the hypothalamus, the 20:80 whey:casein formulation intake was associated with reduced neuronal activity in the supraoptic nucleus (SON) and the ventromedial hypothalamus (VMH). The reduced Fos in these two areas may at first seem counterintuitive. Classically, the SON has been linked with satiety processing as it is targeted by appetite suppressing cocaine-amphetamine-regulated-transcript, CCK and GLP-1 and it releases – among others - anorexigenic oxytocin (Marsset-Baglieri *et al.*, 2014; Stanstrup *et al.*, 2014; Hoefle *et al.*, 2015). However, it should be noted that a greater level of c-Fos immunoreactivity in the SON has been linked with palatable high-sugar diet consumption (Hume *et al.*, 2017). Furthermore, oxytocin has been also suggested to be relevant to hedonic feeding and food preferences, particularly in relation to sugar consumption. As for the VMH, neurons in this area are able to sense glucose, with some being excited by an increase in glucose concentration, while others inhibited by it, the phenomenon specific to subdivisions of this hypothalamic region (Kang *et al.*, 2004). It is important to note that an increase in the activity of VMH neurons has been observed in rats upon sweet taste receptor stimulation with palatable caloric sucrose and non-caloric saccharin solutions (Rao & Prabhakar, 1992). Therefore, it is possible that the higher c-Fos levels observed in the VMH and SON in animals exposed to the 60:40 formula is a consequence of enhanced palatability of the whey-enhanced formulation.

The relative gene expression analyses in the hypothalamus and brain stem following 24-hour exposure to the standard 20:80 versus 60:40 formulation revealed that the 20:80 ratio produced higher mRNA expression levels of anorexigenic genes such as MC3R, OXT and POMC in the brainstem and GLP1R in the hypothalamus. This suggests that consumption of the 20:80 whey:casein formula is associated with changes in expression in the melanocortin, OXT and GLP-1 systems, the key players in ensuring early termination of food intake (and in the case of OXT – a possible interplay between the rewarding and satiating effects of the diets that differ in the whey:casein ratio; a hypothesis to follow up on with direct manipulation of OXT signalling alongside milk diet intake.

3.6 Conclusions

Feeding behaviours in mouse laboratory models is affected with the switch from the 20:80 to 60:40 whey:casein in GM-based formulations. The latter is more readily accepted and preferred in choice scenarios. Altered feeding is accompanied by unique central function following either ratio. Notably, hindbrain activity suggests a strong gustatory and visceral response to formula intake. Different activity and expression of genes in hypothalamic structures suggest heighten satiety signalling with elevated melanocortin, OXT and GLP-1 system function with 20:80 intake – and a potential link of OXT signalling and SON and VMH activity to sweet tastant ingestion.

This set of studies focussed solely on response to GM-derived formulation. However, the variation in whey and casein profiles between GM and CM alters related peripheral processes. GM-specific casein content creating a looser curd in gastric conditions, facilitating easier proteolysis and accelerated gastric emptying compared to CM. Gastric digestion patterns of GM and CM formulations with modified whey:casein also exhibit species difference, with slow digestion of high casein CM formula mitigated in high casein GM formulations. However, comparative description of post-absorptive differences is scarce, with limited description of altered endocrine and circulating nutrient levels following these species milk intake. Chapter 3 extends the current observations of altered feeding with GM formulations to examine response of the mice model to CM-derived formulations with the natural 20:80 and adjusted 60:40 whey:casein ratios. I also re-examine GM preference in cross-species presentations described in Chapter 2, giving animals choice of formulations with the same or different ratio but sourced from different species to explore how species preference is modified with protein fraction adjustments.

3.7 References

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Chapter 4

Whey:casein adjustment in cow's milk from 20:80 to 60:40 affects food intake, brain stem and hypothalamic gene expression and neuronal activation whilst superseding preference for goat's over cow's milk.

4.1 Abstract

As described in Chapter 3, rodents display higher preference for whey-enhanced goat's milk (GM) formulations (i.e., with a 60:40 whey:casein ratio) over a 20:80 whey:casein GM. Changes in feeding-related brain activity (defined through c-Fos mapping) and relative expression of genes accompanied this enhanced preference. However, it is unknown whether the heightened preference for the 60:40 milk would be retained if - instead of a highly palatable and preferred GM - a somewhat less preferred (albeit, still palatable) cow's milk (CM) was used. Thus, in this chapter, I replicated feeding paradigms and analyses of brain gene expression and c-Fos IR with CM formulations with the natural 20:80 and adjusted 60:40 whey:casein ratios. Mice given CM formulations exhibited higher preference for the 60:40 over 20:80 whey:casein CM ratio. This elevated preference from the 60:40 CM was retained even when animals had simultaneous access to the 20:80 GM formulation. Consumption of similar quantities of 20:80 vs 60:40 CM differently affected cFos IR (in the paraventricular, dorsomedial, arcuate and lateral hypothalamic nuclei and in the nucleus of the solitary tract in the brain stem) and relative gene expression (the melanocortin and opioid system transcripts). It can be concluded that the 60:40 whey:casein milk formulations are palatable and they are more preferred regardless of the species from which the milk was derived, indicating that whey enhancement is a potent driver of milk overconsumption. Mechanistic commonalities in the

whey:casein ratio changes in CM versus those described for GM in Chapter 3 include the observed hindbrain neuronal activity changes. Differences in hypothalamic c-Fos IR and gene expression patterns as well as minor differences in no-choice feeding paradigms indicate that the species from which milk was derived modifies some feeding-related processes driven by the shift in the whey:casein ratio.

4.2 Introduction

As described in detail in the introductory section to Chapter 3 of this thesis, not only is the protein content of a diet important, but so is the actual composition of the protein fractions. In milk-based diets, two protein fractions are of particular significance: whey and casein. Apart from their presence alone, it is in fact their ratio that impacts on a number of ingestive and post-ingestive processes related to, among others, gut, (neuro)endocrine, and metabolic functions (Kung *et al.*, 2018; El Khoury *et al.*, 2019; Ye *et al.*, 2019). This altered physiological response to different whey:casein ratios is intuitive, given the outcomes of whey and casein intake are disparate – "fast" whey passes through the stomach and is absorbed quickly producing rapid, high peaks of plasma amino acids accompanied by strong hormonal release (Hall *et al.*, 2003; Veldhorst *et al.*, 2009; Sukkar *et al.*, 2013) whilst "slow" casein clots in the stomach, extending digestion and amino acid absorption and – consequently - producing an (arguably) weaker hormonal response (Mahe *et al.*, 1996; Boirie *et al.*, 1997; Hall *et al.*, 2003; Calbet & Holst, 2004; Bowen *et al.*, 2006; Choi *et al.*, 2009; Veldhorst *et al.*, 2009). Ye *et al.* (2019) observed altered gastric digestion with natural and adjusted milk formulations. Higher casein content in 20:80 whey:casein cow's milk (CM) formulations resulted in larger casein aggregates, slowing gastric digestion of proteins (Ye *et al.*, 2019). Studies involving blood analyses (e.g. Kung *et al.* (2018); El Khoury *et al.* (2019)) have found altered hormonal response following cereal breakfasts served with CM with the natural versus adjusted whey:casein ratios. When given prior to ad libitum lunch, the 60:40 whey:casein milk produced lower blood glucose levels

whereas the 20:80 ratio, lower appetite (Kung *et al.*, 2018). Lower blood glucose was insulin-independent, likely being a result of delayed gastric emptying and post-prandial release of GLP-1, CCK.

Furthermore, changes in whey and casein content affect the brain. It has been shown that post-meal brain processing, particularly in serotonergic pathways, in individuals given milk protein (Choi *et al.*, 2009; McManus *et al.*, 2015; Andreoli *et al.*, 2016; Nilaweera *et al.*, 2017) is modified when whey and casein fractions are given in combination (Diepvens *et al.*, 2008; Potier *et al.*, 2009; Lorenzen *et al.*, 2012; Marsset-Baglieri *et al.*, 2014; Pezeshki *et al.*, 2015). In my data presented in Chapter 3,I found that the whey-enhanced GM was more avidly consumed than the 20:80 GM formulation and that this was associated with unique gene expression and c-Fos immunoreactivity (IR) changes in feeding-related circuits. Specifically, intake of the 20:80 GM formulation downregulated melanocortin receptor 3 (MC3R), pro-opiomelanocortin (POMC) and oxytocin (OXT) transcripts in the hypothalamus and glucagon-like peptide 1 (GLP-1) receptor (GLP1R) mRNA in the hindbrain. Brainstem c-Fos in the rostral and caudal nucleus of the solitary tract (rNTS; cNTS) and hypothalamic ventromedial nucleus (VMH) were impacted by the whey:casein ratio shift.

One has to consider, however, that the appetite and brain processing changes (presented in Chapter 3) upon an adjustment of the whey:casein ratio from the natural 20:80 to 60:40 as well as the previous report on whey/casein effects on the serotonergic system, do not take into account whether a similar response would be achieved if the whey:casein adjustment was done in a milk derived from another species. In other words, a plausible scenario that should be taken into account is that the milk 'vehicle' for whey:casein may affect a plethora of mechanisms induced by the two protein fractions. This is particularly important since GM formulations have different digestion patterns, with natural and adjusted ratios having comparable hydrolysis rates with smaller particulates than the 20:80 CM formulation (Ye *et al.*, 2019). GM has relatively

lower concentrations of α_{s1}-casein, thus forms a looser curd in gastric conditions which is more easily digested (Ambrosoli *et al.*, 1988; Glantz *et al.*, 2010; Logan *et al.*, 2015; Freitas *et al.*, 2019; Wang *et al.*, 2019; Ye *et al.*, 2019). As a consequence, post-absorptive and satiety response to GM intake is unique to that of CM. In line with that, a greater reduction in the desire to eat and subjective hunger has been shown in individuals given GM- than CM-based breakfast (Rubio-Martín *et al.*, 2017), and this outcome was speculated to be related to GLP-1 signalling and circulating triglyceride levels. Furthermore, as described in Chapter 2 of this thesis, central processes and feeding behaviours also differ in response to GM vs CM in laboratory rodents: Mice and rats prefer GM-based diets and expression of several feeding-related genes is affected by consumption of those milk types.

Given that variations in appetite for GM vs CM are underpinned by unique central processing following ingestion of each milk type, in the current Chapter of the thesis, I ask whether the heightened preference for the 60:40 whey:casein ratio in GM reported in Chapter 3 would persist if, instead of the GM, a CM-based formulation was given to the animals. Therefore, this chapter examines the response in animals fed with CM rather than GM formulations with natural 20:80 whey-to-casein ratio (control), vs the adjusted 60:40 ratio to examine (a) whether the modification of the whey:casein ratio affects palatability- and acceptability-related feeding parameters, (b) whether amount-matched consumption of either formulation elicits unique c-Fos IR in feeding-related brain sites, and (c) whether 20:80 vs 60:40 whey:casein CM consumption leads to changes in expression of key regulatory genes in the hypothalamus or brainstem. Finally, animals' preference for GM over CM (described in Chapter 2) was re-evaluated in the context of the two whey:casein ratios to understand whether these ratios supersede the palatability and acceptability of GM.

4.3 Material and Methods

4.3.1 Animals

Adult male C57Bl mice (AgResearch, Hamilton, New Zealand) were single-housed in a temperature-controlled room (22°C) with a 12:12 LD cycle (lights on at 0900). Animals had ad libitum access to standard chow (Diet 86, Sharpes Stock Feed, Wairarapa, New Zealand) and tap water unless stated otherwise. Groups were weight-matched. The studies were approved by the University of Waikato animal ethics committee (ethics approval number 1057).

4.3.2 Milk formulations

CM- or GM-based milk formulations (Dairy Goat Cooperative, Hamilton, New Zealand) had the natural ratio of 20% whey and 80% casein (control solutions: 20:80 CM; 20:80 GM), whereas experimental formulations contained 60% whey and 40% casein (60:40 CM; 60:40 GM). For composition, refer to Table 4.1. Formulations were powdered and reconstituted in tap water immediately before exposure. All animals were pre-exposed to the diets prior to the feeding trials to prevent neophobia.

Table 4.1: Nutritional composition of prepared CM and GM formulations per 100 mL

	kJ	Protein (g)	Whey protein (%)	Fat (g)	Carbohydrate (g)
20:80 CM	286.5	1.6	20.0	3.8	7.3
60:40 CM	273.5	1.4	60.0	3.4	7.2
20:80 GM	278.1	1.3	20.0	3.5	7.5
60:40 GM	275.5	1.4	60.0	3.5	7.1

4.3.3 Feeding studies

CM-based formulation treatment paradigms for adult mice are outlined in

Table 4.2

Table 4.2: Schematic of CM-based formulation treatment paradigms in adult mice

	 Sated	 Deprived	 Adult	 Mouse
Single tastant				
CM formulations - 1 hour	X		X	X
GM formulations - 24 hour	X		X	X
Choice				
CM and/or GM formulations 2 bottle choice	X		X	X

4.3.3.1 Preference for simultaneously presented CM formulations

Mice (n=7/group) were acclimatised to the two-bottle presentations in two separate sessions one week prior to the trial. On the experimental day at 10:00, chow and water were removed from cages and mice were given access to two bottles, containing either 20:80 or 60:40 CM formulations. Formulation intake after two hours was measured in grams.

4.3.3.2 Preference of CM or GM formulations with 20:80 or 60:40 whey:casein ratios

The two-bottle scenario in Section 4.3.3.1 was repeated, with mice (n=7/group) receiving access to CM or GM formulations with the control vs adjusted whey:casein ratio (20:80 CM vs 60:40 GM; 60:40 CM vs 20:80 GM) or with the same whey:casein ratio (20:80 CM vs 20:80 GM; 60:40 CM vs 60:40 GM). Formulation intake after two hours was measured in grams.

4.3.3.3 Intake of CM formulations in a 1-hour exposure paradigm

Mice (n=8/group) had water and chow removed from cages at 08:00, and animals were then given a single bottle of control 20:80 adjusted 60:40 CM for one hour. Formulation intake was measured in grams.

4.3.3.4 Intake of individually presented CM formulations in a 24-hour exposure paradigm

On the experimental day, both chow and water were removed (at 09:00) and a bottle containing either the control 20:80 or the whey-adjusted 60:40 CM formulation was placed in the cage

(n=8/group). The formulations were the only source of both calories and fluid for the next 24 hours. Afterwards, intake was measured in grams.

4.3.4 Neuronal activation in feeding-related hypothalamic and brainstem areas after consumption of the same amount of the 60:40 versus 20:80 CM formulation.

The purpose of this experiment was to assess whether consumption of the same amount of the 20:80 vs 60:40 whey:casein CM milk formulation (as described in described in 4.3.3.1 and akin to the c-Fos experiment presented in the previous chapter pertaining to GM) induces a different pattern of neuronal activation in the hypothalamus and brain stem, areas that are crucial in the regulation of food intake.

One hour after exposure to the 20:80 or 60:40 CM formulation, mice were anaesthetised with 35% urethane i.p. Animals were perfused with saline (20 mL) followed by 50 mL of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Brains were removed and postfixed overnight in PFA at 4 °C. Coronal 60 μm sections created via vibratome (Leica, Germany) were processed for c-Fos immunostaining. Sections were incubated in 3% H_2O_2 in 10% methanol (in TBS; pH 7.4) for ten minutes, then overnight in rabbit anti-Fos antibody (1:3000; Synaptic Systems, Goettingen, Germany) at 4°C. Tissues were incubated in goat-anti-rabbit secondary antibody (1:400; Vector Laboratories, USA) then in avidin-biotin complex (Vector Laboratories, USA) at room temperature for an hour each. Peroxidase was visualised with 0.05% DAB, 0.01% H_2O_2 and 0.2% nickel sulfate (Sigma, USA). All incubation solutions utilised 0.25% gelatin and 0.5% Triton X-100 (Sigma, USA) in TBS. Intermediate washes were in TBS. Sections were mounted on gelatinised slides, dried and dehydrated with ascending concentrations of ethanol followed by xylene and subsequently embedded with Entellan (Merck, Germany). c-Fos IR nuclei were counted manually and bilaterally in all

regions (4-5 sections/animal) by a person blinded to group allocations at 10x and 40x magnifactions on Nikon microscope. Densities recorded as c-Fos positive nuclei/mm^2 were averaged per group.

4.3.5 Hypothalamic and brainstem gene expression following 24-hour exposure to the 20:80 CM vs 60:40 CM formulations

Following 24-hour individual exposure to 20:80 or 60:40 CM formulation that results in the consumption of the same amount of the milk solutions (as described in section 4.3.3.4, n=8/group), mice were sacrificed via cervical dislocation at 09:00 and the brain stem and hypothalamus were dissected and stored for one hour in 1mL RNAlater (Invitrogen, USA) at room temperature and then at -80°C until processing.

Thawed samples were transferred from RNAlater into TRIzol (Life Technologies, USA; 1mL/100mg tissue). Following mechanical homogenisation, chloroform was added (0.2ml/100mg tissue). Samples were centrifuged for 20 minutes, 10,000× *g* at 4 °C. The clear phase was siphoned and RNA was precipitated with addition of 0.5mL of isopropanol with 10minute ice bath incubation. Samples for were centrifuged again at 4°C for 20 minutes at 10,000× *g.* Pellet was retained and washed in 0.3mL of ethanol and centrifuged at 4°C for 10 minutes at 10,000× *g.* The ethanol was removed and pellet air dried.

1 µL of DNAse buffer (dNature, New Zealand), 1 µL DNAse (dNature, New Zealand) and 8 µL of DEPC water was added to the pellet and incubated at 37°C for 30 minutes, followed by 67°C for 10 minutes with 1 µL stop buffer (dNature, New Zealand). Absence of DNA was confirmed with HOT FIREPol Blend Master Mix (dNature, New Zealand) PCR and agarose gel electrophoresis. RNA concentrations were determined with a nanodrop.

Reverse transcription synthesised cDNA with iScript Advanced cDNA synthesis kit (BioRad, New Zealand) and was confirmed with PCR and gel electrophoresis.

RT-qPCR determined relative expression of housekeeping genes (ActB, β-tubulin, H3B) and genes of interest. Reaction mixes containing 4 µL of 25 ng/µL sample cDNA, 1 µL each of forward and reverse primers (5 µM), 10 µL iTaq Universal SYBR Green Supermix (BioRad, New Zealand) and 4 µL MilliQ water. Reaction were run in duplicate with MilliQ water negative controls for each primer pair. Amplification protocol was 95 °C for 15 minutes, followed by 45 cycles of 15 seconds at 95°C, 15 seconds at the primer-specific annealing temperature and 30 seconds at 72°C. Primers sequences used are detailed in Table 4.3.

4.3.6 Statistical analyses

Unpaired Student's *t* test for two-group comparisons was used to analyse food intake and immunohistochemistry data. Analyses of qPCR data were performed with BioRad CX Manager software (BioRad, New Zealand), followed by unpaired Student's *t* test. Differences were considered statistically significant at $p < 0.05$.

Table 4.3: Forward and reverse primers for housekeeping and target genes used in RT-qPCR analyses of hypothalamic and brainstem relative gene expression following CM formulations

Gene	*Forward*	*Reverse*
ACTB	5′-AGTGTGACGTTGACATCCGT-3′	5′-TGCTAGGAGCCAGAGCAGTA-3′
BTUB	5′-CGGAAGGAGGCGGAGAGC-3′	5′-AGGGTGCCCATGCCAGAGC-3′
H3B	5′-CCTTGTGGGTCTGTTTGA-3′	5′-CAGTTGGATGTCCTTGGG-3′
MC4R	5′-CTTATGATGATCCCAACCCG-3′	5′-GTAGCTCCTTGCTTGCATCC-3′
POMC	5′-GACACTGGCTGCTCTCCAG-3′	5′-AGCAGCCTCCCGAGACA-3′
NPY	5′-GGTCTTCAAGCCGAGTTCTG-3′	5′-AACCTCATCACCAGGCAGAG-3′
KOR	5′-CACCTTGCTGATCCCAAAC-3′	5′-TTCCCAAGTCACCGTCAG-3′
MOR	5′-CCTGCCGCTCTTCTCTGG-3′	5′-CGGACTCGGTAGGCTGTAAC-3′
DYN	5′-GACAGGAGAGGAAGCAGA-3′	5′-TCAGAGCCAGTAAGCCAAGCA-3′
OXT	5′- CCTACAGCGGATCTCAGACTG-3′	5′-TCAGAGCCAGTAAGCCAAGCA-3′
ORX	5′-GCCGTCTCTACGAACTGTTGC-3′	5′-CGCTTTCCCAGAGTCAGGATA-3′
PNOC	5′-AGCACCTGAAGAGAATGCCG-3′	5′-CATCTCGCACTTGCACCAAG-3′

OPRL1	5′-ATGACTAGGCGTGGACCTGC-3′	5′-GATGGGCTCTGTGGACTGACA -3′

4.4 Results

Mice given a choice between the 20:80 and 60:40 whey:casein ratio CM formulations more avidly consumed the whey-enhanced formulation (P<0.0001,Figure 4.1). This was also observed in milk cross-species (i.e., GM vs CM) simultaneous presentations of formulations with different whey:casein ratios (20:80 CM vs 60:40 GM; 60:40 CM vs 20:80 GM), where the 60:40 ratio was preferred regardless of species from which milk was derived (P<0.0001; Figure 4.2AB). When presented with formulations of the same whey:casein ratio (20:80 CM vs 20:80 GM; 60:40 CM vs 60:40 GM), GM formulations were preferred, significantly so for the 60:40 GM over 60:40 CM (P=0.0112) and with a trend approaching significance in the case of the 20:80 CM/GM combination (P=0.1210; Figure 4.2CD). In non-choice scenarios involving 1-hour and 24-hour tastant availability, there was no difference in the intake of 20:80 and 60:40 CM formulations (Figure 4.3).

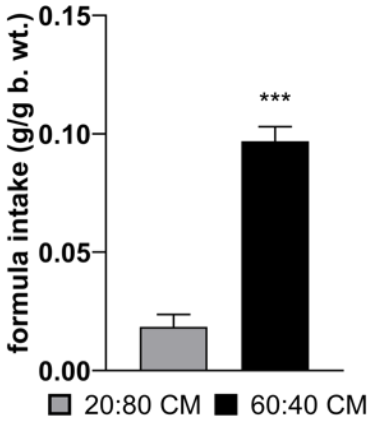

Figure 4.1 Non-deprived animals consume less of the 20:80 CM whey:casein formulation than of the 60:40 CM formulation during a 2-h episodic exposure of simultaneously presented diets. *** P ≤ 0.001.

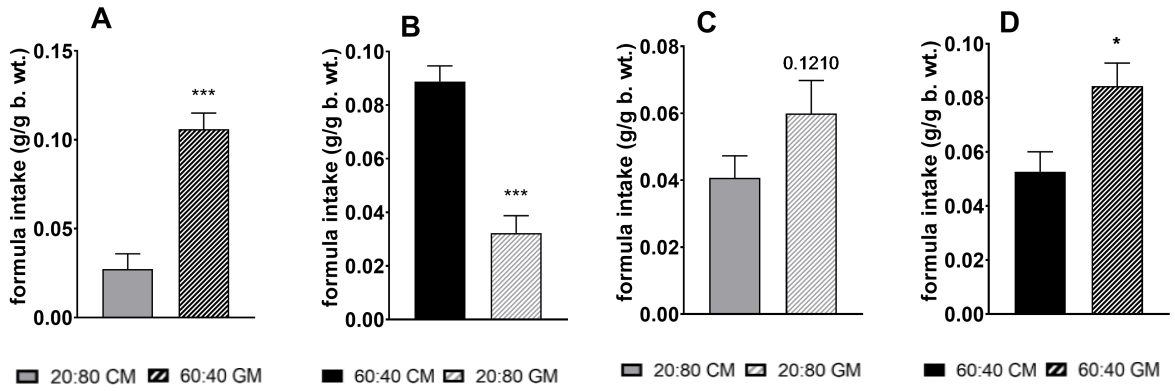

Figure 4.2: Two bottle choice tests of CM or GM formulations. In milk cross-species presentation with different whey:casein ratios (A: 20:80 CM vs 60:40 GM; B: 60:40 CM vs 20:80 GM), the adjusted 60:40 ratio formulations were consumed avidly regardless of species. In cross species presentation with the same whey:casein ratios (C: 20:80 CM vs 20:80 GM; D: 60:40 CM vs 60:40 GM), the GM formulation were preferred in 60:40 choice. * - $P \leq 0.05$; *** $P \leq 0.001$

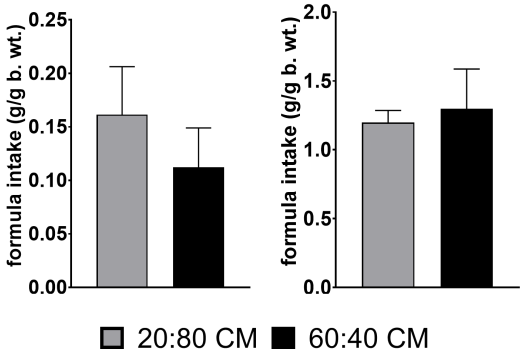

Figure 4.3: Individual presentation of cow's milk formulations for 1-hour (left) and 24-hour (right) produced similar intake of control 20:80 and whey-adjusted 60:40 CM formulations.

Intake of a similar amount of 20:80 vs 60:40 CM formulation over a 1-hour meal affected c-Fos IR in 6 out of 13 sites following one-hour exposure to 20:80 or 60:40 CM formulations (Figure 4.4). Animals that consumed the 60:40 formulation had fewer c-Fos positive nuclei in the hypothalamic paraventricular nucleus (PVN; P=0.0046), dorsomedial hypothalamic nucleus (DMH; P=0.024), arcuate nucleus (ARC; P<0.001), lateral hypothalamic area (LHA; P<0.001), and in the caudal nucleus of the solitary tract (cNTS; P=0.0063) compared to the 20:80 whey:casein CM-fed conspecifics. Higher c-Fos IR in the 60:40 than 20:80 whey:casein CM group was found in the rostral portion of the NTS (rNTS; P=0.0081).

Mice consuming the 60:40 whey:casein CM formulation for 24 hours had increased brainstem expression of the melanocortin receptor 3 (MC3R; P=0.006) and opioid-like receptor 1 (OPRL1, P=0.005) genes as well as reduced hypothalamic expression of neuropeptide Y (NPY) (Figure 4.5). Decreases in hypothalamic melanocortin receptor 4 (MC4R; P=0.067) and orexin (ORX; P=0.056) expression following 60:40 CM approached, but did not reach, significance.

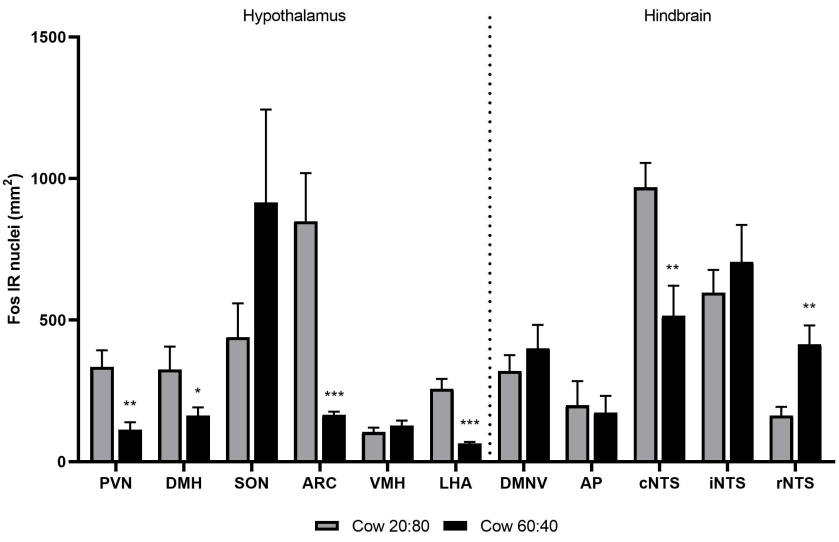

Figure 4.4: cFos immunoreactivity was higher in the rostral nucleus of the solitary tract (rNTS) and lower in the paraventricular nucleus (PVN), dorsomedial hypothalamic nucleus (DMH), the arcuate nucleus (ARC), lateral hypothalamic area (LHA) and caudal nucleus of the solitary tract (cNTS) following intake of the CM formulation with the natural 20:80 whey:casein (20:80

CM) compared to whey-adjusted 60:40 whey:casein ratio (60:40 CM). SON – supraoptic nucleus; VMH – ventromedial hypothalamic nucleus; NacS – nucleus accumbens shell; NacC – nucleus accumbens core; DMNV – dorsal motor nucleus of the vagus; AP – area postrema; iNTS – intermediate nucleus of the solitary tract; * - $P \leq 0.05$; ** - $P \leq 0.01$; *** - $P \leq 0.001$.

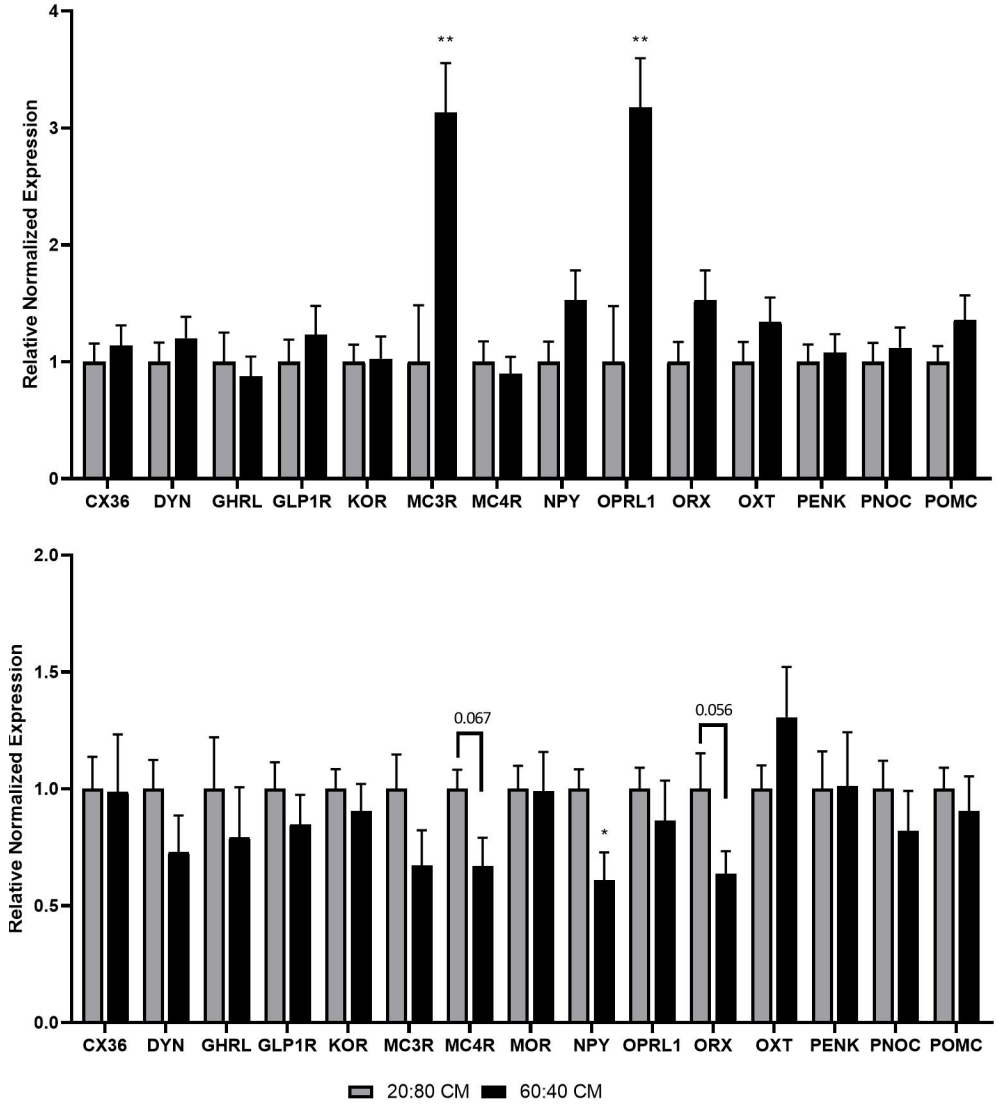

Figure 4.5: Brain stem (top) and hypothalamic (bottom) relative gene expression following 24 hour consumption of the control 20:80 vs 60:40 CM formulation. 60:40 CM formulation intake was associated with increased expression of the melanocortin receptor 3 (MC3R) and opioid-like receptor 1 (OPRL1) mRNA in the brainstem and reduced neuropeptide Y (NPY) gene expression in the hypothalamus. * - $P \leq 0.05$; ** - $P \leq 0.01$.

4.5 Discussion

Data presented in the previous Chapter 3 showed that laboratory mice more avidly consume GM with the adjusted 60:40 compared to the natural 20:80 whey:casein ratio and that this is associated with unique changes in feeding-related circuitry in terms of c-Fos immunoreactivity and expression of feeding-related genes. The studies included in the current Chapter expand on these findings by determining that the whey-enhanced ratio is more preferred to 20:80 even in another type of milk, namely in the bovine formulation, the milk known for gustatory and ingestive characteristics distinct from GM. Acute appetitive impacts with the shift from the natural to adjusted ratio in the CM-based formulation are accompanied by changes in neuronal activity in feeding regulatory regions and in relative expression of genes in relevant signalling pathways.

Milk, including CM, is a palatable tastant and animals readily consume it even in the absence of energy needs (as shown, e.g., in Chapter 2). However, this set of data indicates that a whey:casein ratio modification in the protein fraction of CM affects preference for CM. In the feeding experiment that involved a choice between CM formulations containing the 20:80 vs 60:40 whey:casein ratios, laboratory mice consumed approximately four times more of the whey-adjusted CM than of the natural 20:80 formulation. This profound preference for the 60:40 CM was achieved in the paradigm that relied on merely 2 hours of the formulation presentation, thereby reflecting a greatly heightened drive to consume this solution. This level of the difference is indeed high: for example previous studies employing short-term, simultaneous presentations of isocaloric and isopalatable liquids containing fat vs sugar or different types of sweet solutions have shown differences within 10% of the consumed volume (Olszewski *et al.*, 2010; Herisson *et al.*, 2014), and a potent pharmacological treatment specifically aimed at affecting preference was able to make animals consume twice as much of one solution than the other (Herisson *et al.*, 2014). Elevated consumption of the 60:40

formulation over the 20:80 ratio was similarly observed with GM-based formulations in Chapter 3, where mice also strongly preferred the adjusted GM formulation to a similar degree. Repetition of this pattern reaffirms the importance of whey:casein ratio on the palatability of milk formulations regardless of the species source.

It should be noted that in the feeding experiment involving a non-choice presentation of CM containing either 20:80 or 60:40 whey:casein, we did not see a significant difference in consumption. However, a difference in intake is less frequently seen in paradigms that rely on a single tastant presentation than in choice paradigms. This is the case even with highly palatable tastants (e.g. sweet and fat solutions (Olszewski *et al.*, 2010; Herisson *et al.*, 2014)) or in fasted, and thus highly motivated, states (Kimura *et al.*, 2003).

The fact that short-term intake volumes of individually presented formulations were similar to the total volume consumed in choice paradigms as well as to the short-term palatable sucrose solution intake in Chapter 2 and in previous studies from our laboratory (Gartner *et al.*, 2018), indicates that CM formulations regardless of their whey:casein ratio are palatable. Interestingly, unlike the GM-based 60:40 whey:casein formulation which was consumed in greater quantities than 20:80 even in the non-choice scenario, significantly so after 24 hours (see Chapter 3), this effect was absent in CM. This may suggest the additive effect of the more palatable whey:casein ratio and – as shown in Chapter 2 – the more preferred milk type (GM), was apparent through enhanced consumption even in the paradigm that did not involve a choice.

Importantly, in cross-species milk formulation comparisons, the preference for GM over CM, observed with skim milks in Chapter 2, persisted somewhat with formulations with the same whey to casein ratio. Mice presented with the formulations derived from GM and CM with adjusted 60:40 ratio consumed significantly more of the GM formulation. A similar but nonsignificant trend for GM preference was observed with the 20:80 formulations, an

interesting observation as the protein composition of the 20:80 formula more closely resembles that of the skim milks in Chapter 2 that were not adjusted for protein content. It highlights the potential role of fat (both that was added in formulations and that was removed with skim milks) making species milks more palatable. It should be emphasised that alteration in whey:casein ratio supersedes preference derived from the species from which milk was sourced. When mice were provided with formulations of different whey and casein content, they displayed more avid consumption of the 60:40 GM or CM formulations over the alternative species 20:80 option. Most notably, whey:casein shifted the GM over CM preference with avid 60:40 CM formulation consumption over that of the 20:80 GM. This indicates the whey:casein ratio is a potent driver of consumption (and, potentially, overconsumption) of milk. Despite the potential synergistic effect between the milk type and whey:casein driving intake in non-choice paradigms, whey:casein appears to have the most critical impact on the preference for these solutions.

Taking into account differences in preference for the 60:40 vs 20:80 whey:casein CM formulations, it is not surprising that consumption of similar amounts of each of the tastants resulted in a different response of brain circuitry in terms of c-Fos immunoreactivity (IR) as well as gene expression.

The analysis of c-Fos IR in the hindbrain revealed that the animals given a more preferred 60:40 whey:casein CM formulation had a higher level of neuronal activation in the rostral portion of the NTS (rNTS). Elevated activation of this area has been associated with oral delivery of flavoured tastants, e.g., sweet sucrose, bitter quinine, or sour citric acid (Harrer & Travers, 1996; King *et al.*, 1999; Travers, 2002; Stratford *et al.*, 2017), and therefore it is quite plausible that the 60:40 whey:casein CM elicits a more profound sensory response at the hindbrain level. On the other hand, c-Fos in the caudal portion of the NTS (cNTS), which integrates visceral input relayed by vagal efferents (Horst *et al.*, 1989; Rinaman, 2010), was

lower after the 60:40 whey:casein CM intake. One can therefore speculate that the combination of sensory and visceral processing at the NTS level contributes to elevated intake of the 60:40 whey:casein CM. Importantly, the combined findings from this chapter and from Chapter 3 indicate that NTS c-Fos IR in response to the 60:40 vs 20:80 milk is the same regardless of the species from which the milk was derived. Therefore, the NTS may be a common denominator for elevated consumption of formulations with the enhanced whey content.

Several hypothalamic regions displayed suppressed c-Fos IR after 60:40 whey:casein CM consumption. Most notably, it was observed in the PVN, which receives rich cNTS input and hosts neurons synthesising a number of anorexigenic peptides including oxytocin and CRH. And it was approximately five-fold lower in the ARC, which encompasses cells producing an appetite suppressant alpha-MSH. This might potentially link a propensity to ingest greater amounts of the 60:40 whey:casein CM with downregulation of mechanisms that prevent overeating. The 60:40 whey:casein CM also produced a less robust c-Fos response in the DMH, which has been linked with CCK-driven appetite suppression (Bellinger & Bernardis, 1984; Kobelt *et al.*, 2006), as well as well as with the LHA which mediates cannabinoid-and orexin-mediated hyperphagia (Thorpe *et al.*, 2006; Perez-Morales *et al.*, 2012) and appetite-suppressing GABAergic signalling (Turenius *et al.*, 2009).

One should note that unlike the NTS c-Fos IR which was – as shown in this chapter and in Chapter 3 – the same in both CM and GM formulations, hypothalamic c-Fos mapping produced different results depending on the species from which milk was sourced. It can likely be contributed to the combined effect of the whey:casein ration and the unique characteristics of GM vs CM (as delineated in Chapter 2).

Finally, the qPCR analysis of transcript levels in the brainstem and hypothalamus underscores a unique functional relationship between the feeding-relevant mechanisms and whey:casein ratio in the CM protein fraction. Most notably, consumption of the 60:40 whey:casein CM was

associated with a strong trend toward decrease in the anorexigenic MC4R expression in the hypothalamus and significant upregulation of the receptor for orexigenic nociceptin/orphanin FQ in the brain stem, which is in line with the more avid intake of the 60:40 formulation. Surprisingly, expression of hypothalamic NPY and orexin was lower, whereas brain stem MC3R was higher in the whey-enhanced formulation group, however, it may be either unrelated to feeding (e.g., in the case of ORX, it may be associated with wakefulness) or it may reflect a combined effect of species source and whey:casein content on broader post-ingestive peripheral mechanisms (which is likely considering some alignment between GM vs CM-induced gene expression, e.g., in ORX and MC transcripts).

In conclusion, laboratory mice display preference for the whey-enhanced CM formulation, and this phenomenon is underpinned by changes in brain neuronal activity and gene expression.

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Chapter 5
General Discussion and Perspectives

Food provides nutrients needed for the proper functioning of cells, tissues and, consequently, of the organism. Food intake has to therefore meet the body's energy and nutrient requirements. As such, feeding behaviour is regulated by neural and endocrine activity which promotes consumption during energy or nutritional deficit and suppresses feeding upon meeting those demands. The GI tract and other secretory organs and tissues, such as the pancreas and adipose tissue, release hormones that – appropriate for the fed and fasted state – modify/adjust feeding behaviour. Peripheral factors relevant to energy balance influence brain activity through direct interaction with centrally expressed receptors for circulating nutrients and hormones (both orexigenic and anorexigenic) or through vagal projections to the hindbrain. This leads to upstream activity and molecular changes within central pathways which regulate feeding. Alongside processes regulating energy homeostasis, palatability of ingestants elicits signalling in the reward system, as well as, via an interconnected network, in broader feeding regulatory circuits targeted by projections from reward areas.

Milk or milk-based foods are near ubiquitous in Western diets. Although milk, from an evolutionary point of view serves as a food during early life, it is commonly incorporated into adult diets, significantly contributing to daily energy intake and being a source of both nutrients and palatability. This can either be in close-to-natural forms of milk or in milk formulations and milk-based products that are modified to target certain health-related outcomes, i.e., lactose-free hypoallergenic milks, milk-based weight loss or high-protein sport drink diets, and whey-enhanced formulations. In addition to the issue that milk gets incorporated into our dietary repertoire in such different forms, one should consider an oftentimes neglected fact that the composition and physical characteristics of milks derived from different species vary, too. These differences are so profound that, as delineated in the Introduction to this thesis,

consumption of milk sourced from different species, e.g., goats versus cows, generates unique post-ingestive processes including digestion kinetics and endocrine responses. For example, comparing CM - the most commonly consumed, and therefore studied, milk type in Western societies – with GM, shows digestion differences related to protein fractions. While similar in total protein content, GM and CM vary in whey and casein (Park *et al.*, 2007; Wendorff *et al.*, 2017). GM contains higher β-casein and α_{s2}-casein and reduced α_{s1}-casein concentrations (Grosclaude & Martin, 1997; Carillier-Jacquin *et al.*, 2016; Wendorff *et al.*, 2017). In gastric conditions, casein micelle structure collapses leading to coalescence of casein proteins into a curd (Jenness, 1980). Lowered α_{s1}-casein content in GM produces a looser curd (Ambrosoli *et al.*, 1988), facilitating easier casein proteolysis (Barbé *et al.*, 2013) and more rapid emptying of GM casein products into the intestinal lumen than CM (Maathuis *et al.*, 2017; Hodgkinson *et al.*, 2018). Variation in absorption rate influences subsequent endocrine cascade and appetite, with Rubio-Martín *et al.* (2017) reporting a GM breakfast with semi-skimmed milk, cheese and white bread lowering a desire to eat and hunger more so than a CM breakfast, also associated with altered GI GLP-1 release.

The data obtained in the course of my experimental work show for the first time that GM is ingested more avidly than CM by mice and rats and that this phenomenon has physiological underpinnings in terms of feeding-relevant neural responses. As the studies were conducted in two standard laboratory species (rats and mice) and – moreover – in animals that belonged to distinct age categories (adolescent, adult, aged), the consistent feeding outcomes lend credence to generalising the conclusion regarding preference for GM over CM across key laboratory animal models. The set of studies in Chapter 2 (Palatability of goat's versus cow's milk: insights from the analysis of eating behaviour and gene expression in the appetite-relevant brain circuits in laboratory animal models**)** bridges some of the gaps in the current literature regarding how a species from which milk was sourced, specifically bovine and caprine milk, affects intake and preference for this milk. I found that, while both GM and CM are highly

palatable, GM is preferred and more readily consumed. Increased intake and preference for GM appears to be due to a higher hedonic value rather than impaired/delayed satiety, as suggested by expression of reward- and satiety-related genes.

Both CM and GM were found to be palatable across no-choice paradigms in my experimental work. When presented separately in animals being in a fed or fasted state, consumption of milks was comparable to the palatable positive control of a sweetened solution. Consumption of these three palatable liquids exceeded that of the "bland" cornstarch. High palatability of milks was also confirmed with solid diets enriched with milk. Laboratory rodents showed elevated consumption of milk-containing chows compared to standard ('bland') chow during episodic presentations. Both milks were also highly palatable across the three age groups examined (adolescent, adult and aged) with elevated intake of CM and GM equivalent to the palatable sweetened water. While the aged animals exhibited slightly lowered intake of palatable solutions than the adult or adolescent groups, one should note that in both humans and non-human animals, aging is associated with an increased prevalence of anhedonia (Lampe *et al.*, 2001), and that includes reduced consumption of palatable diets (Herrera-Pérez *et al.*, 2008; Shin *et al.*, 2012; Inui-Yamamoto *et al.*, 2017; Sakai *et al.*, 2017). While intake for palatability was indeed mildly suppressed in the aged rats and mice, they still displayed elevated intake of milk, which indicates that the animals retain sensitivity to the pleasant consequences of milk consumption despite the overall lowered hedonic responsiveness to foods during old age. Though the results have been obtained in animal studies, they nonetheless serve as an important clue for possible human interventions in that a palatable and nutritive tastant, namely milk, can be treated as a dietary tool helping in mitigating anhedonic consequences of aging.

Whilst both milks were found to be palatable, this research also identified differences in the context of preference. In regard to relative palatability, simultaneous presentations of GM and CM diets (liquid formulations and solid chow) underscored a near-uniform preference for GM.

Preference for GM in a liquid form was higher than that for CM in mice and in rats, with the only exception being adolescent mice that ingested similar amounts of GM and CM. In simultaneous presentations of CM- and GM-enriched chows, adult and aged rats and adult mice preferred goat milk-enriched pellets; aged mice showed a trend toward GM preference. Lickometer feeding patterns strongly support the notion of the elevated GM preference, with four-fold increase of licks of skim GM within the first five minutes of exposure, persisting with a two-fold increase in the following five minutes. Licking microstructure analysis showed a high bout number and an extended bout length for goat milk. The observed increase in the initial GM intake, bout number and duration parallels licking patterns of highly palatable solutions (Davis & Smith, 1992; Davis & Perez, 1993; Spector *et al.*, 1998; Glass *et al.*, 2001), suggesting that heightened palatability of GM drives the intake of this milk type.

Despite both CM and GM being common foods on a global scale our understanding of potential differences in palatability and acceptability of these milks is extremely limited and relies mainly on anecdotal evidence. It appears that, prior to this experimental work, no other laboratory animal study utilising CM and GM diets had directly compared preference for and relative intake of these milks. Nor have human studies performed any in-depth analysis of participants' preferences for GM versus CM, other than providing very limited notions, such as that GM is perceived as novel with a unique "goaty" taste (Mowlem, 2005; Park & Haenlein, 2013, Rubio-Martín *et al.*, 2017; Milan *et al.*, 2018). It should be emphasised though that in the context of human observations preferences are strongly biased by habituation. The aforementioned human studies were conducted in geographic locations in which CM is predominantly consumed (Western diet) and, therefore, where exposure to CM is extremely unlikely to constitute a new experience, whereas GM may more often be met with unfamiliarity. Foods with familiar gustatory perception (flavours and textures) are generally preferred (Torrico *et al.*, 2019; Cheng *et al.*, 2020). Habituation is a nearly unavoidable confounding factor in human study designs, needing either cohorts naïve to or with equal

exposure to both milk types. Considering this, the use of animal models in the current studies allowed us to examine whether differences in preference for GM vs CM exist at all in individuals (animals) whose prior exposure to both milk types could be controlled and, thus, devoid of the habituation bias. In fact, the very consistent preference for GM across various models in our experiments is so striking that – taking into account the mainly anecdotal evidence of a somewhat negative perception of GM in human subjects with lifetime exposure to primarily Western diets - it may be of interest to examine whether early-life habituation to CM or GM in laboratory rodents would also affect their GM-vs-CM preference later in life. This might be a worthwhile endeavour especially in light of the data showing that animal milk-based formula intake in early infancy modifies later dairy preference in humans (Mennella & Beauchamp, 2002; Maslin *et al.*, 2016). One might even speculate – though this hypothesis is quite premature - that the onset of a preference for a given milk may occur already during the pre-weaning phase: rodent maternal diet during lactation is known to affect food preferences once the pups reach adult age (Carlin *et al.*, 2013; Gugusheff *et al.*, 2015).

Accordingly, it should be acknowledged animals used in this study consumed their dams milk as neonates. Rodent milk differs in composition from the test diets examined here, varying across species and strain (Treadway *et al.*, 1986; Del Prado, *et al.*, 1997; Bautista, *et al.*, 2021; Boumahrou *et al.*, 2009, Godbole, *et al.*, 1981; Görs, *et al.*, 2009; Ragueneau, 1987). Notability, whey:casein ratio is near the 20:80 ratio and a β-casein ratio closer to CM (Wendorff *et al.*, 2017; Boumahrou *et al.*, 2009). No controls for pre-programming of preferences concerning milk diets conveyed during the neonatal period are used in these studies. Potentially, this could be achieved with earlier postnatal interventions where manual milk administration replaces milk supply from dams and later life feeding patterns and preferences are evaluated in the offspring. This would ascertain whether preferences observed in these studies are indeed confounded by neonate exposure to dams' milk.

The analyses of brain gene expression accompanying intake of GM and CM found disparate effects of the consumption of each milk type at the central level. Intake of GM induces more robust activation of hedonic pathways, possibly indicating that increased intake and preference for GM over CM is related to reward. Alongside this, in pathways involved in feeding for energy GM promoted expression of satiety genes and suppression of hunger-related genes, thus suggesting that the heightened intake of GM was not due to abnormal hunger-satiation processing. It is well known that reward-driven intake is mediated by interconnected regions activated following ingestion of palatable foods, from lower brain stem areas to higher order orbitofrontal cortex, ventral palladium and NAcc (Berridge & Kringelbach, 2015). Following palatable food intake, dopaminergic, opioid and endocannabinoid signalling in these areas is enhanced, and so is the expression of genes that encode relevant neurotransmitters, their receptors and associated signalling factors. Opioid signalling is associated with hedonic stimulation, especially the "liking" component of reward-driven consumption (Berridge & Kringelbach, 2015), and an increase in the opioid tone promotes intake of palatable tastants, from the simple, single-macronutrient, calorie-dilute ingestants to the preferred, highly caloric and nutritionally complex foods (Lynch, 1986; Giraudo *et al.*, 1993; Levine *et al.*, 1995; Weldon *et al.*, 1996; Peciña & Berridge, 2000; Glass *et al.*, 2001; Naleid *et al.*, 2007; Olszewski *et al.*, 2011). Dopamine signalling within the mesolimbic system (originating in the ventral tegmental area and encompassing ventral striatal structures including the NAcc (Pandit *et al.*, 2011)), produces the "wanting" of a rewarding tastant (Wyvell & Berridge, 2000), and connexin-36 (Cx36) mediates synchronicity within dopamine neuronal networks (Steffensen *et al.*, 2011). Importantly, the results presented here show that GM consumption increased expression of mu (MOR) and kappa (KOR) opioid receptors in the hypothalamus, and also caused a trend toward their upregulation in the NAcc and brainstem. Additionally, dopamine receptor transcript levels were affected following GM, alongside the changes in Cx36 expression. Auxiliary modulation of the reward system by GM might also be linked with other

feeding-related transcripts in the NAcc, including GHSR, PNOC and OPRL (Perelló & Zigman, 2012; Skibicka *et al.*, 2013; Hardaway *et al.*, 2016; Statnick *et al.*, 2016; Hernandez *et al.*, 2021). Thus, the response of the reward system to GM is relatively vast, involving a diverse repertoire of genes and, importantly, these findings are congruent with the outcomes of food intake experiments which found GM to be more palatable/preferred than CM.

As mentioned above, gene expression studies indicate that GM consumption does not affect expression of hunger- and satiety-related genes adversely, i.e., in a manner that would suggest impaired hunger-satiety processing in response to GM exposure. Upregulation of anorexigenic melanocortin system-associated genes, MC3R and MC4R, as well as reduced expression of orexigenic NPY and ORX transcripts following GM intake is expected considering that milk provides calories (Levine *et al.*, 2004; Nixon *et al.*, 2012). Furthermore, elevated OXT expression following GM indicates both GM palatability (as OXT is typically released with intake of palatable food, especially that containing carbohydrates) and maintenance of a satiety response (Amico *et al.*, 2005) (Miedlar *et al.*, 2007) (Mullis *et al.*, 2013; Herisson *et al.*, 2014).

Our meal microstructure lickometer analysis supports this notion of preserved satiety with GM intake with similar timeframes of attenuation of consumption between GM and CM suggesting that elevated preference for GM was not due to delayed satiation (Glass *et al.*, 2001). In line with this finding, prior studies in human subjects report no deficit in satiety to GM or CM diets. Milan *et al.* (2018) reported equivalent perceived satiety and appetite as well as circulating levels of appetite-regulating hormones in participants given fortified GM or CM drinks. Rubio-Martín *et al.* (2017) even suggest that GM-based breakfast heightens satiety compared to CM breakfast: the subjects in that study reported a reduced desire to eat and subjective hunger, and those questionnaire data were elegantly paralleled by GLP-1 and triglyceride levels. Overall, higher intake of GM does not stem from impaired satiety processing and can be attributed to

palatability and the consequences at the reward system's level may be viewed as a promising parameter for possible translational applications of this finding to human dietary interventions.

While it is clear, at least in animal models, that GM is more preferred than CM, it is important to reiterate the fact that Western diets rarely rely on unmodified milk, but rather utilise milk products that have undergone processing. These modifications are likely to affect a number of characteristics of milk, leading to altered palatability and acceptability. Therefore, one cannot declare a priori that all GM-based milk formulations are more palatable and more avidly consumed than CM-derived tastants. Considering this crucial caveat, I continued my work on feeding responses to CM and GM by focusing on formulations that had incorporated one of the most common compositional adjustments: the natural 20:80 whey:casein ratio found in bovine and caprine milks was whey-enhanced to 60:40 in order to match the typical whey:casein ratio of human milk. In Chapter 3 (Changes in feeding and related brain function in mouse model following consumption of infant formulations with variable whey and casein content) and Chapter 4 (Whey:casein adjustment in cow's milk from 20:80 to 60:40 affects food intake, brain stem and hypothalamic gene expression and neuronal activation, and it supersedes preference for goat's over cow's milk), I furthered GM and CM comparisons with formulations differing in the whey:casein ratio.

That a shift in whey:casein may significantly impact intake of milks that differ in this parameter seemed quite intuitive considering a disparate impact that each of the fractions has on eating-related behavioural and physiological processes. Whey and casein fractions have unique digestion, with "fast" whey passing through the stomach and into the intestine whilst "slow" casein aggregating there. The resulting absorption features early and high peaks in plasma amino acids with whey and slower, lower and longer elevated levels with casein. Endocrine response to whey has been described as more pronounced. Long-term preliminary studies on whey and casein intake published prior to the commencement of this research have shown a

possible link with serotonergic and melanocortin signalling. Interestingly, when whey and casein are consumed together, GI endocrine release is unique to that seen with isolates and not necessarily proportional to either fraction's contribution (Diepvens *et al.*, 2008; Lorenzen *et al.*, 2012). Studies that have examined the acute digestive, endocrine and appetitive responses to natural and adjusted whey:casein ratios indicate that such modifications influence satiety-related processes. Ye *et al.* (2019) demonstrated *in vitro* that elevating casein content in CM increased micelle aggregate size thereby slowing 60:40 CM formula digestion. 20:80 and 60:40 GM formulations, having similar particle size and digestion rates, differed in the rate of protein digestion and in amino acid plasma spikes (Dangin *et al.*, 2001; Juvonen *et al.*, 2011), particularly notable with casein (Juvonen *et al.*, 2011). Appetite and endocrine functions following natural and whey-adjusted CM formulations were examined in studies by Kung *et al.* (2018) and El Khoury *et al.* (2019). Following formulation intake as part of a cereal-based breakfast, the 60:40 ratio produced a lower plasma glucose peak (Kung *et al.*, 2018). Glycaemic regulation was insulin-independent, relating rather to gastric emptying speed altered by GI hormone release, modifying the rate of glucose absorption and thereby blood glucose levels (El Khoury *et al.*, 2019). Furthermore, human subjects reported reduced appetite with the 20:80 formulation (Kung *et al.*, 2018).

The observations in humans are strongly supported by the outcomes of the studies presented in this thesis. In the feeding studies in Chapters 3 and 4, I observed that preference was heavily impacted by whey:casein ratios. Chapter 3 utilised GM-based formulations with the natural 20:80 and adjusted 60:40 whey:casein ratios whilst Chapter 4 examined response to CM-based formulations with the same ratios. In choice scenarios, mice avidly consumed the adjusted 60:40 formulations over 20:80 with both GM- and CM-derived formulations. The four-fold increase in 60:40 intake compared to the 20:80 formulation indicates a strong influence of whey:casein ratio on palatability, given that in previously published choice paradigms using highly palatable tastants of sugar and fat, intake disparity is within 10% of the total volume

(Olszewski *et al.*, 2010; Herisson *et al.*, 2014). This high palatability of the 60:40 whey:casein is reaffirmed in the choice paradigms in Chapter 4. When mice were given simultaneously two milk formulations of the same whey:casein ratio but from a different species source (i.e. CM 20:80 vs GM 20:80 or CM 60:40 vs GM 60:40), animals preferred GM formulations, consistent with preferences observed in Chapter 2 with skim milks. However, this species source-based milk preference was superseded by the whey:casein ratio when animals were given alternate ratios from two different species. Specifically, when given a choice between the CM formulation with the 60:40 ratio and the GM formulation with the 20:80 ratio, animals avidly consumed the CM formulation despite GM preference in the whey:casein matched scenarios.

The data in the single tastant tests are largely consistent with the notion of heightened palatability of 60:40 milks. In Chapter 3, mice given a single bottle of GM (either 60:40 or 20:80) for 24 hours had higher intake of the 60:40 formulation whilst in Chapter 4, though mice given a single bottle of either 60:40 or 20:80, CM had equal intake in the same paradigm with CM formulations. This suggests that palatability of GM and of the 60:40 ratio could have a additive effect, promoting elevated consumption above that of the two CM formulations. This may explain why, in the cross-source milk presentations in Chapter 4, there was the significant preference for 60:40 GM over 60:40 CM, while there was only a trend for 20:80 GM over 20:80 CM preference. It is also possible, given the lack of fats in the skim milk comparisons in Chapter 2, that inclusion of species milk lipids or added fats in formulations may change palatability of these species milks.

One of the questions that may arise based on the results of these studies is whether whey enhancement is therefore a desirable modification of milk content. It should be considered that GM is already palatable and, therefore, its intake is associated with a rewarding value that by itself is sufficient to promote consumption. Thus, when choosing a diet with the aim of elevating food intake, replacing CM with GM might be an optimal intervention. This is because

by combining either CM or GM with whey enhancement (and thus departing from the natural whey:casein ratio and elevating feeding even further), an increase in calorie intake may be so high that it would produce a greater risk of bringing about unwanted metabolic consequences through delivering a diet with more of an obesogenic potential. This issue certainly needs to be investigated in-depth in future studies involving long-term exposure to milks that differ in whey:casein ratios to assess their impact on energy homeostasis.

One should also note that a departure from a natural 20:80 whey:casein ratio in milk is not without consequences for brain activation and for gene expression. Though CM and GM formulations were examined by me in two separate projects (Chapter 3 and 4) dedicated to each milk type (either CM or GM) makes direct statistical comparison impossible, there are clear commonalities and differences that can be attributed to whey:casein ratio and to the species source of a given milk, respectively. In fact, the nucleus of the solitary tract (NTS) appears to be a common denominator for neuronal activity changes induced by whey enhancement. Regardless of whether GM or CM, the intake of the whey:casein 60:40 milk formulation increases c-Fos IR in the rostral portion of the NTS (rNTS) and decreases the density of c-Fos-positive neurons in the caudal portion of this nucleus (cNTS). Given whey and casein fractions have such unique digestive and absorption kinetics, it is intuitive that adjusting the ratio would elicit activity in the cNTS as this area is responsive to visceral input (including vagal) as well as to the circulating nutrient status (Horst *et al.*, 1989; Johnson & Gross, 1993; Rinaman, 2010). The rNTS activity is quite interesting as it aligns well with the findings showing differences in palatability/preference between the whey:casein ratios from our animal experiments as well as from some human studies utilising a formula (Kung *et al.*, 2018) or yoghurt (Tomaschunas *et al.*, 2012; Cheng *et al.*, 2019). Importantly, the rNTS receives gustatory and sensory input and displays reactivity to specific tastes (Harrer & Travers, 1996; King *et al.*, 1999; Travers, 2002; Rinaman, 2010; Stratford *et al.*, 2017).

Interestingly, the hypothalamus was the region where c-Fos mapping after 20:80 vs 60:40 formula intake produced a differential pattern of activity that was dependent on the species from which a milk was sourced. In GM-fed animals, the whey-enhanced formulation affected c-Fos only in the SON and VMH. On the other hand, in the CM experiment, I found a change in neuronal activation in the PVN, LHA, DMH and ARC, and the change in activity was opposite depending on the species (in GM, whey enhancement caused an increase, whereas in CM, a decrease in c-Fos immunoreactivity). c-Fos observations are accompanied by changes in relative gene expression in the two studies. Transcript level changes in the hypothalamus between groups given 60:40 vs 20:80 milks are notably different in the CM study than in the GM study, whereas the brainstem profiles have at least some similarities between the two studies (e.g., in melanocortin receptor, orexin or dynorphin genes – showing either significance or trend approaching significance). Considering the role of the hypothalamus in energy homeostasis control, these data should be therefore viewed as an additional impetus to conduct long-term studies that focus on potential metabolic consequences of whey enhancement. Furthermore, these outcomes serve as evidence that a departure from the natural whey:casein ratio in milk is not a neutral change in terms of altering short-term feeding responses as well as the relevant central mechanisms that govern ingestive behaviour.

5.1 Limitations

This work has highlighted new findings regarding control of food intake following milk consumption, specifically variations in feeding behaviour and central processes with different milk types. These are the first studies to examine such outcomes of the context species source and common modifications in milk proteins made in manufacture of milk diets in a systematic way. However, it is important to note the limitations of these studies conclusions.

Several limitations are noted including the translation of rodent studies to humans. There are benefits for animal models in dietary research compared to humans, such as high level of

control over factors impacting intake and metabolic processes such as lifestyle, genomic variability and diet compliance, and the availability of techniques allowing for closer examination physiological processes, such as invasive tissue collection. However, innate differences in biology between our species and that of laboratory models does limit extrapolation from diet and metabolic studies (Lai, *et al.*, 2014; Chalvon-Demersay, *et al.*, 2017; Hintze, *et al.*, 2018; Suleiman, *et al.*, 2020). The use of animal models has eliminated the biases in preferences occurring with pre-exposure one would expect within a population through use of animal models. Whilst this gives methodological accuracy for unbiased milk species preferences, this may not necessarily translate to human populations as perceptions of novel foods, especially milks, are biased by what is standard within a culture (Torrico *et al.*, 2019; Cheng *et al.*, 2020; Mennella & Beauchamp, 2002; Maslin *et al.*, 2016; Mowlem, 2005; Park & Haenlein, 2013). Therefore, observed preferences in intake and related changes in neuronal activity and gene expression may not necessarily represent any given human population due to difference in biology between species and innate preferences of any given individual.

Alongside this, the most obvious confounding factor in milk preferences studies would be giving rodents lactose-rich diets as they exhibit lactose intolerance following weaning (De Angelis, *et al.,* 1984; Labrie, *et al.*, 2016). Rats have displayed poor digestibility and intolerance of diets with higher lactose contents around 20-30% (De Angelis, *et al.,* 1984; van De Heijning, *et al*., 2015). Given lactose content of test diets used here had high lactose content, rodent lactose intolerance could impact the palatability of these diets. Lactose intolerance studies examined chronic intake of high lactose, noting the outcomes of lower absorption, altered carbohydrate and lipid metabolism and increased incidence of diarrhoea (De Angelis, *et al.,* 1984; Alexandre, *et al.*, 2013; van De Heijning, *et al*., 2015). However, acute impact of short periods of lactose intake is relatively unknown. Diet studies were preceded with brief pre-exposure to the diets to prevent hyponeophagia and milk diets were avidly consumed and

highly palatable, comparable to sweet sucrose solutions. This would suggest that animals did not experience malaise after the pre-exposure session, as they did not display aversion for the diets. Indeed, high palatability was observed across all age groups examined in Chapter 2, notable as intestinal lactase in rodents decline with age and would make the any negative post-prandial effect more pronounced (Labrie, *et al.*, 2016). This suggests minimal negative side effects from lactose intake on palatability, but this could be confirmed with conditioned taste avoidance studies with milk diets, ensuring no malaise results from short episodic intake.

Another limitation to be considered in these studies is use of appropriate controls. Suitable control diets that account for complex macronutrient profile of milk diets as well as for variations that occur in naturally sourced diets are difficult to achieve. Controls used in Chapter 2 for the preference studies included sucrose and cornstarch whilst gene expression studies utilised isovolumetric water intake. The former served as palatable and unpalatable caloric controls. Near equal intake to sucrose and higher intake than cornstarch solution was noted. This pattern persisted even in low energy, food deprived state, indicating that these skim milks were highly palatable and not just rewarding for their caloric content. Water served as a control for the gene expression study in Chapter 2. It serves as a non-caloric control, accounting for gene expression associated with intake volume, thirst abolition and gastric distension (Traub, *et al.*, 1996; Sun, *et al.*, 2006; Tang, *et al.*, 2006; Sabbatini, *et al.*, 2008), given its isovolumetric consumption. However, water as a control does not account for changes resulting from nutritive components of diets, where isocaloric solutions and compositionally matched diets may serve as better controls. These nutritive components are harder to control for, as the test diets in all three chapters have complex macronutrient profiles which are naturally sourced.

An isocaloric solution of sucrose or cornstarch, or other commonly used carbohydrate solutions such as glucose, may provide a caloric control but would not accurately match metabolic outcomes of lactose (Alexandre, *et al.*, 2013; Mohammad, *et al.*, 2011; Roser, *et al.*, 2009;

Krishna, *et al.*, 2020) nor would these solutions, or a lactose or galactose control, account for the changes that would come from consumption of the other macronutrients present in the milk solutions. One could consider other milks or milk-based diets as controls, such as rat and human milks, or commercially available infant formulations and milk shake replacement diets. These solutions have complex blend of nutrients more closely resembling test diet composition compared to single nutrient solutions. However, these would not account for effects of species macronutrient differences or added ingredients in different milk-based diets.

Another aspect constricting controls is the natural variability in milk composition, with changes in composition dependent on genetics and animal breed (Scholtens, *et al.*, 2020; Bainbridge, *et al.*, 2016; Lim, *et al.*, 2020), health status (Al-Farha, *et al.*, 2017; Gonçalves, *et al.*, 2020; Novac & Andrei, 2020), lactation period (Zhang, *et al.*, 2020; Kljajevic, *et al.*, 2018), fodder intake and nutrient supplementation during lactation (Thoh, *et al.,*2017; Murney, *et al.*, 2019; Muir, *et al.*, 2015; Ariza, *et al.*, 2019) and environmental changes (Kljajevic, *et al.*, 2018; Bertocchi, *et al.*, 2014). Accurately reflecting these variations in a macronutrient-matched control is near impossible.

Given the limitations of accurate controls for milk diets, direct comparison of formulations were made in Chapters 3 and 4.

Compositional variation also contributes to flaws in these studies regarding replicability and validity. Milks used here are pooled from multiple animals and farms which may counterbalance some of the aspects influencing composition described about, though may still vary from batch to batch. This highlights issues in study replicability faced by all naturally sourced diet studies. Additionally, there are some variations in test diets macronutrient composition used in Chapters 3 and 4. Whilst these studies aimed to manipulate whey and casein contents of the milk diets, there were other changes in composition such as a ~10% difference in fat content in the CM formulations, a ~13% and ~7% difference in total protein

content as well as a ~1% and ~5% carbohydrate difference in the CM and GM formulations respectively. Whilst these changes are smaller in comparison to the intentional changes in whey and casein contents of these diets (~90% and ~78% difference in whey and casein, respectively, in CM formulations, ~105% and ~60% difference in whey and casein, respectively, in GM formulations), these variations could impact brain activity and gene expression profiles (Hu, *et al.*, 2018). Therefore, a role of other macronutrient variations in changes attributed to whey and casein manipulation cannot be discounted.

Another component that could be influencing histological and gene expression outcomes is intake volume. Gastric distension influences activity in the hypothalamus and brain stem (Traub, *et al.*, 1996; Sun, *et al.*, 2006; Tang, *et al.*, 2006; Sabbatini, *et al.*, 2008) as will as caloric load (Lazzarino, *et al.*, 2017; Lazzarino, *et al.*, 2019; Xu, *et al.*, 2007). There was not a significant difference in intake of skim milks (or water control) or of CM-based formulations prior to histological testing and gene expression analyses in Chapters 2 and 4. While these studies were not strictly calorie controlled, it is unlikely that intake volume would have greatly impacted the results from these chapters. However, in Chapter 3, whilst 2-hour GM-formulation consumption for histological analyses had similar intake, the 24-hour intake of the 60:40 formula was significantly larger than the 20:80 formula. Interestingly, higher caloric intake should induce higher expression of satiety-related genes, however, the 20:80 formula had reduced expression of anorexigenic POMC and OXT. It is unclear, then, how intake volume has influenced gene expression in this chapter. Follow up studies are attempting to control for this variability from ad libitum consumption through intragastric delivery of isovolumetric boli.

5.2 Conclusions

The overarching aim of this doctoral research was to examine whether laboratory rodents display different preference for GM versus CM-based diets and whether it is associated with

different responsiveness of brain systems controlling eating for energy and/or reward. The findings of these studies are:

- GM and CM liquid and solid diets are highly palatable.
- GM is preferred over CM by rats and mice and across different age groups (adolescent, adult and aged).
- GM intake elevates expression of dopaminergic and opioidergic genes in the NAcc, suggesting a functional relationship between GM intake and reward.
- GM intake also upregulates some melanocortin system genes, indicative of a link with satiety processing.
- Whey enhancement is associated with elevated intake of and preference for milk regardless whether sourced from goats or cows.
- Whey:casein ratio manipulation supersedes impact of a species from which the milk was sourced on the preference for a formulation.
- Intake of 20:80 and 60:40 whey:casein milk formulations is accompanied by alterations in neuronal activity and gene expression in key regions regulating food intake
- Both CM and GM formulations induce hindbrain activity associated with gustatory and visceral sensations, relating to manipulating the whey:casein ratio.
- Differences in gene expression and activity of hypothalamic nuclei following GM or CM formulations indicate that a species from which the milk was sourced impacts food intake-related processes.

5.3 References

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Chapter 6

Appendix

6.1 Composition of CM- and GM-enriched chow

Base Skim Milk Powder Composition (1% Milk Fat)	Goat Skim Milk Powder (SMP)		Cow SMP	
	%	g/kg	%	g/kg
Protein	37.8	120	37.1	120
Fat	1.7	5.40	0.86	2.78
Ash	9.4	29.84	7.3	23.61
Moisture	4.1	13.02	4.2	13.58
Lactose	47	149.21	50.54	163.47
Total	100	317.46	100	323.45
	Goat SMP		**Cow SMP**	
Skim Milk Chow—Ingredient	g/kg		g/kg	
Goat SMP—to supply 12% protein	317.46			
Cow SMP—to supply 12% protein			323.45	
Vitamin mix	50.00		50.00	
Salt mix	50.00		50.00	
Corn oil (added to make 5% fat)	44.60		47.22	
Starch	447.15		452.80	
Lactose (added to make 16.5%)	15.79		1.53	
Cellulose	75.00		75.00	
Moisture				
	1000.00		1000.00	
Target Dietary Content				
Protein	12%		12%	
Fat	5%		5%	
Lactose	16.50%		16.50%	
Fiber	7.50%		7.50%	
Starch	44.7%		45.3%	
Calories				
Protein (4 cal/g)	48		48	
Fat (9 cal/g)	45		45	
Lactose (4 cal/g)	66		66	
Fiber (2 cal/g)	15		15	
Starch (4 cal/g)	179		181	
Calories/100 g	353		355	
% calories				
Protein	14%		14%	
Fat	13%		13%	
Lactose	19%		19%	
Fiber	4%		4%	
Starch	51%		51%	
Skim Milk Powder (1% Milk Fat)	**Goat SMP**		**Cow SMP**	
Ingredients	g/kg		g/kg	
Goat SMP	317.46			
Cow SMP			323.45	
Vitamin mix	50.00		50.00	
Salt mix	50.00		50.00	
Corn oil	44.60		47.22	
Starch	447.15		452.80	
Lactose	15.79		1.53	
Cellulose	75.00		75.00	
Moisture				
	1000.00		1000.00	

Base Skim Milk Powder Composition (1% Milk Fat)	Goat Skim Milk Powder (SMP)	Cow SMP
Target Dietary Content		
Protein	12%	12%
Fat	5%	5%
Lactose	16.50%	16.50%
Fiber	7.50%	7.50%
Starch	44.7%	45.3%
Calories		
Protein (4 cal/g)	48	48
Fat (9 cal/g)	45	45
Lactose (4 cal/g)	66	66
Fiber (2 cal/g)	15	15
Starch (4 cal/g)	179	181
Calories/100 g	353	355
% calories		
Protein	14%	14%
Fat	13%	13%
Lactose	19%	19%
Fiber	4%	4%
Starch	51%	51%