Welfare aspects of stocking density in farmed rainbow trout, assessed by behavioural and physiological methods

PhD Thesis

Written by Danielle Caroline Laursen
Defended 27 May 2013

DTU Aqua
National Institute of Aquatic Resources

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Technical University of Denmark

National Institute of Aquatic Resources

Section for Aquaculture

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Preface

This PhD study was funded partly by DTU Aqua and partly by a project my supervisor Erik Höglund was awarded funding for from the Danish Ministry for Food, Agriculture and Fisheries.

The PhD study took place between March 2010 and March 2013. During these 3 years, there were several requirements to fulfill in order to complete the PhD degree at DTU Aqua. The experimental work was carried out at DTU Aqua, Section for Aquaculture in Hirtshals, where the two-tank facility used for the experiments was built especially for this project. I disseminated my work at several international conferences and workshops as oral and poster presentations, and have written manuscripts to be published in scientific peer reviewed journals. I was also fortunate to spend some time abroad at the other Institutions, at Pertti Panulas' Group at the University of Helsinki, Neuroscience Center and the Integrative Behavioural Biology Group of Rui Oliveira at University Institute in Lisbon. These research stays were funded by generous donations from BeFINE and the Idella Foundation respectively. To obtain my ECTS credits, I attended several courses offered at DTU and other Universities in Denmark and abroad.

This PhD thesis is the last requirement to be fulfilled for obtaining my PhD degree from DTU Aqua. It is also the end result of 3 years of hard work!

There are many people that have to be acknowledged for their guidance and support in realising this achievement.

Firstly, I would like to express my gratitude to my supervisor Erik, not only for guiding me through this process, but also for giving me this opportunity. It has been a pleasure to be your PhD student, through the good and the hard times.

I would also like to thank my co-supervisor Peter for his valuable input in this thesis.

I extend my thanks to my former colleagues at DTU Aqua in Hirtshals:

The Section for Aquaculture. I am appreciative to: Alfred, Ole, Rasmus and Erik P. for their practical assistance with the fish. Bodil for advice and help in the lab and with the experimental facility. Ulla, Dorthe and Brian for assistance in the lab. Per for advice and support. Grethe for endless help with administration;

The PhD school in Charlottenlund. A special thank you to Nina for her support, especially towards the end;

My former PhD colleagues in Hirtshals – Maria M., Paulo, Jordan – I will remember the good times. To my former office mates Patricia and Marine, to Madelene and to Maria R-G, thank you for the fun, the support and friendship.

Finally, I am indebted to my family and friends:

My mama, sister, and brother who stand by me always. My father, to whom I would like to dedicate this work. My amazing friends for cheering me on.

Last, but not least, thank you to my dearest Benedikt for his patience and encouragement over the last three years.

To anybody who I have been so absent minded as to have missed in this list, I thank you!

On to the next chapter in my career, over and out.

Caroline Laursen

Caroline Laursen

Alloa, February 2013

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Summary

There is an increasing amount of interest in the welfare of fish from aquaculture. There are several aquaculture practices that may act as chronic stressors and therefore have the potential to negatively impact welfare. Stocking density has been highlighted as a particular welfare concern, from both an ethical and practical point of view.

A quantity of research has been conducted on the relationship between stocking density and indicators of welfare in farmed rainbow trout *Oncorhynchus mykiss*. The studies to date have revealed that both low and high densities have the potential to detrimentally affect welfare in rainbow trout. Several studies have endeavoured to make specific recommendations for maximum stocking density limits for rainbow trout. However, wide discrepancies exist, highlighting the fact that it has been a challenge to identify density limits that promote optimal welfare and production in rainbow trout. This emphasises the significance of developing alternative methods that provide insight into the potential density limits that are optimal for welfare and performance in rainbow trout.

Here, a behavioural method using two-tank systems was developed and applied. The two-tank systems consisted of two identical tanks which were attached to each other with a doorway allowing the fish to move freely between the two tanks. By studying the spatial distribution of fish in two-tank systems stocked with different densities and the neuroendocrine stress levels of the fish, a density level was established that showed indications of crowding. The results revealed that a level of aversion to crowding had been reached at an absolute density of approximately 140 kg m^{-3}.

Additionally, the influence of the established density limit on physiological indicators of welfare and performance were investigated. At this density of 140 kg m^{-3}, the lower oxygen consumption rates and lower quantity of scale loss collected from the tanks suggested reduced levels of social hierarchy related aggressive encounters. Higher brain serotonergic activity in the brain stem of individuals held at this density indicated elevated stress levels, despite low concentrations of plasma cortisol. The reduced energetic expenditure at 140 kg m^{-3} resulted in a better utilisation of ingested feed and hence growth performance.

Taken together, despite the chronic stress levels at this density, the results showed that at this density the reduced energy expenditure, attributed to reduced aggressive social interactions, resulted in a better growth performance. Therefore, it may be concluded that

application of the method using the two-tank systems provided new insight into an optimal stocking density limit for rainbow trout. Furthermore, the method presented here provides a promising tool for investigating stocking density levels in rainbow trout. Further development of the current method would consider it applicable for determining limits for a range of culture situations.

Dansk resume

Der er en stigende interesse for dyrevelfærd ved fiskeopdræt. Flere arbejdsrutiner og fremgangsmåder ved opdræt udgør potentielle kroniske stressfaktorer og forårsager måske derfor negativ velfærd. Biomassetætheden ved opdræt er fremhævet som en af de vigtigste faktorer med indflydelse på velfærd, både set fra et dyreetisk og et praktisk synspunkt.

Der er tidligere foretaget undersøgelser af sammenhængen mellem tæthed og mål for velfærd i opdrættede regnbueørreder, *Oncorhynchus mykiss*. Hidtil har det været påvist at både lave og høje tætheder har en potentielt negativ indflydelse på velfærd hos regnbueørred. Adskillige studier har forsøgt at etablere anbefalinger for grænseværdier af tæthed ved opdræt af ørred. Imidlertid er der store variationer i disse anbefalinger, og det er fortsat en udfordring at bestemme tæthedsgrænser for optimal velfærd og produktion af regnbueørred. Dette faktum fremhæver vigtigheden af at forbedre forståelsen af mekanismerne i denne problemstilling.

I dette ph.d. forløb er en to-tanks opstilling blevet udviklet og brugt til at iagttage forsøgsfisks opførsel. Opstillingen bestod af to identiske tanke forbundet med en åbning, der tillod fiskene fri passage. Ved at observere den rumlige fordeling af fisk i dette to-kammer system under forskellige tæthedsgrader, samt målinger af neuroendokrine stress niveauer af fiskene, har det været muligt at opnå en tæthedsgrad der udviste tegn på for høj tæthed (crowding). De opnåede resultater peger på at der opstår et mætnings niveau ved tæthed på omkring 140 kg m^{-3}.

Yderligere er det i denne ph.d. blevet undersøgt, hvorledes den fundne grænseværdi påvirker de fysiologisk indikatorer for velfærd og vækst. Ved det fundne niveau på 140 kg m^{-3}, var der et lavere specifikt iltforbrug og en mindre forekomst af skæltab, tydende på en formindsket forekomst af aggressive interaktionerne mellem fiskene. Der blev fundet højere serotoninaktivitet i hjernen hos individer ved høj tæthed, hvilket indikerer et forhøjet stress niveau, selvom plasma kortisol værdier ikke var forhøjede. Det formindskede energiforbrug hos fisk ved 140 kg m^{-3} tyder på en bedre udnyttelse af den indtagne føde, og dermed bedre tilvækst.

Samlet set viser resultaterne, til trods for tegn på forøget stress, at høje tætheder hos regnbueørred fører til et mindre energiforbrug hos fiskene. Denne observation tilskrives en

reduktion i aggressiv adfærd mellem individer, og udmøntede sig i forbedret vækst hos fiskene. Det kan konkluderes at metoden med brug af to-tanks systemet til at fastsætte grænseværdier for tæthed er valide, og giver fornyet indsigt i problematikken omkring tætheder hos opdrætsfisk. Videreudvikling af metoden vil gøre den anvendelig ved en række forskellige akvakultur situationer.

List of papers

I. Title: *Utilising spatial distribution in two-tank systems to investigate a threshold for crowding in rainbow trout Oncorhynchus mykiss*.

   Authors: Danielle Caroline Laursen, Madelene Åberg Andersson, Patricia I.M. Silva, Erik Petersson and Erik Höglund

   Journal: Applied Animal Behaviour Science (2013) 144, 163-170

II. Title: *Increased aggressive behaviour at low stocking density and chronic stress at higher stocking densities in farmed rainbow trout Oncorhynchus mykiss: indications from oxygen consumption rates, fin erosion, scale loss and neuroendocrine measures*.

   Authors: Danielle Caroline Laursen, Patricia I.M. Silva, Bodil K. Larsen and Erik Höglund

   Journal: Physiology & Behavior *Revision submitted*

III. Title: *Elevated energy expenditure results in reduced growth performance in farmed rainbow trout Oncorhynchus mykiss reared at low density*.

   Authors: Danielle Caroline Laursen, Bodil K. Larsen, Peter V. Skov and Erik Höglund

   Manuscript In prep

   Journal of intended submission: Aquaculture

1. Introduction

In aquaculture, fish may experience a range of unfavourable environmental conditions, such as frequent handling, transport, food deprivation, poor or fluctuating water quality parameters and physical crowding (Ashley, 2007; Huntingford et al., 2006). Crowding is a consequence of intensive fish farming at high rearing densities, which has attracted considerable attention in recent years (Ashley, 2007; Huntingford et al., 2006). This has been the consequence of increasing public concern for the welfare of fish from aquaculture (Huntingford et al., 2006) and recognition by the commercial and scientific communities that inappropriate densities can contribute to reduced welfare in fish (Ellis et al., 2002).

A diversity of research has been conducted on the relationship between stocking density and welfare in farmed rainbow trout *Oncorhynchus mykiss*. This research has been carried out by investigating the effects of varying density levels on indicators of welfare such as, performance, condition, health and stress levels (Boujard et al., 2002; Ellis et al., 2002; Larsen et al., 2012; McKenzie et al., 2012; North et al., 2006; Person-Le Ruyet et al., 2008; Skøtt Rasmussen et al., 2007). Through such studies, it has been possible to draw general conclusions about the influence of rearing density on welfare. There is evidence that inappropriate densities can affect growth and mortality rates, can be detrimental to health, can influence stress levels, can cause fin erosion and gill damage, can increase aggressive and abnormal behaviours and can contribute to deteriorating water quality (reviewed by Ellis et al., 2002). The general perception is that welfare decreases with increasing density. However, research has not reached an unequivocal conclusion on the effects of increasing rearing densities on indicators of welfare. Furthermore, it has been suggested that low densities also potentially compromise welfare (Boujard et al., 2002; Ellis et al., 2002; Larsen et al., 2012; McKenzie et al., 2012; North et al., 2006; Person-Le Ruyet et al., 2008; Skøtt Rasmussen et al., 2007).

Several studies on the correlations between rearing density and fish welfare have used their results to make specific recommendations for maximum stocking densities for rainbow trout, based on their experimental results (Ellis et al., 2002). Depending on the type of rearing system, the recommendations ranged from 4 to more than 267 kg m^{-3} (Ellis et al., 2002). Such a wide range of recommendations highlights the fact that it has been a challenge to identify density limits that promote optimal welfare and production in rainbow trout. The recommendations for optimal stocking from a welfare perspective continue to be ambiguous. Therefore, it is relevant to develop alternative methods that provide insight into the density that are optimal for welfare and performance in rainbow trout. The spatial distribution of animals in a rearing environment might provide useful information on space requirements and preferred stocking density (Dawkins, 2004; Turnbull et al., 2008).

The overall objective of my PhD project was to develop and apply a novel behavioural method for investigating density limits that fish experience as aversive to determine maximum density limits and to study the influence of these density limits on indicators of welfare and performance in farmed rainbow trout. This was achieved by studying the spatial distribution of fish in two-tank systems stocked at different densities, to establish a density level that showed indications of aversion to crowding. The two-tank systems used to carry out the experimental work consisted of two identical tanks which were attached to each other with a doorway, allowing the fish to move freely between the two tanks and distribute themselves accordingly. Additionally, the influence of the established critical density limits on indicators of welfare and performance were investigated.

In this synopsis of my PhD project, I will discuss the background and motivation for carrying out this work, briefly describe the methodology used, provide a summary of the findings obtained and discuss the major conclusions and future perspectives of this work.

2. Background

2.1. Animal welfare

Animal welfare is a complex and contentious topic and continues to be difficult to define (Huntingford and Kadri, 2009; Huntingford and Kadri, 2008). Most definitions of welfare, used by the scientific community, can be divided into three categories which express different ideas about the conditions that consider an animal to be in a state of good welfare: *feelings-based*, *nature-based* and *function-based* definitions (reviewed by Duncan and Fraser, 1997; Fraser et al., 1997; Huntingford et al., 2006; Huntingford and Kadri, 2008).

Feelings-based definitions identify an animal to be in a state of good welfare if the animal "…is free of negative experiences such as pain, fear and hunger and has access to positive experiences, such as social companionship…" (Huntingford and Kadri, 2009; Huntingford and Kadri, 2008). Such definitions imply that even though an animal is healthy, it may not necessarily experience good welfare unless this generates positive feelings. Likewise, an animal that is injured may not necessarily experience bad welfare unless this generates negative feelings (Huntingford and Kadri, 2008).

Nature-based definitions identify an animal to be in a state of good welfare if the animal "…is able to lead a natural life, expressing the same kinds of behaviour as it would in the wild, and is able to meet what are often called its 'behavioural needs'…" (Huntingford and Kadri, 2008). These definitions imply that if animals can show their natural behaviour they experience good welfare and if they cannot express their natural behaviour they experience bad welfare (Huntingford and Kadri, 2008).

Function-based definitions identify an animal to be in a state of good welfare if the animal "…can adapt to its environment and is in good health, with all its biological systems working appropriately…" (Huntingford and Kadri, 2008). These definitions imply that if an animal is in good health and has proper functioning of bodily systems, they are experiencing good welfare. On the other hand, if an animal has bad health and has impairment to bodily functioning, they are experiencing bad welfare (Huntingford and Kadri, 2008).

Each approach reflects different aspects of animal welfare (Huntingford and Kadri, 2009; Huntingford and Kadri, 2008) that provide complementary interpretations of welfare in a given situation (Lawrence, 2008). As suffering (*feeling-based*), impairment of natural behaviour (*nature-based*) and health problems (*function-based*) are co-dependent, the different approaches will often reach the same conclusions about animal welfare (Duncan and Fraser, 1997; Huntingford et al., 2006).

The way animal welfare is measured will depend on how it is defined (Huntingford and Kadri, 2009) (Table 1; After Huntingford and Kadri, 2008). For example, studying welfare using nature-based definitions would focus on assessment of how the behaviour of the farmed animal deviates from how it would behave in the wild. On the other hand, studying welfare using function-based definitions would focus on assessment of physical and physiological status of an animal (Huntingford and Kadri, 2009).

Table 1. Summary of the most commonly used measures of welfare and how they related to the different definitions of welfare (After Huntingford and Kadri, 2008).

		Approach	
Welfare measure	*Feelings-based*	*Nature-based*	*Function-based*
Physical condition			
Injury and disease			•
Immune system functioning			•
Nutritional condition			•
Growth		•	•
Reproduction		•	•
Physiological status			
Metabolic state		•	•
Hormones		•	•
Brain biochemistry	•	•	•
Genes on/off		•	•
Behavioural status			
Behavioural signs of stress/fear	•	•	•
Stereotypes	•	•	
Natural repertoire	•	•	

With regards to assessing fish welfare, the most commonly used measures are those defined by the function-based approach (Huntingford et al., 2008). Using this approach, the physical and physiological conditions of an individual are important indicators of welfare (Table 1), with measures of: injury and disease, immune function, nutritional status, growth performance, reproductive systems, metabolic status, hormonal status, and brain biochemistry.

In this PhD project, the main focus has been on using the function-based definition of welfare, incorporating both physical and physiological elements to assess fish welfare in relation to rearing density. Additionally, one aspect of the nature-based definition was also incorporated, when investigating (natural) aggressive behaviour in relation to stocking density.

2.2. Stress

Stress is a central issue in fish welfare (Ashley, 2007) as chronic activation of the stress response in fish has been demonstrated to have potential maladaptive consequences affecting welfare (Huntingford et al., 2006). Indicators of the physiological stress response are therefore commonly used in welfare studies (Ashley, 2007).

2.2.1. The stress response in fish

The stress response in fish is initiated by a series of neuroendocrine events that function to re-establish homeostasis when confronted with an environmental challenge (Ashley, 2007; Ellis et al., 2002; Ellis et al., 2012; Pottinger, 2008) and can be observed at three different levels: the *primary*, the *secondary* and the *tertiary* stress response (Ellis et al., 2012).

The *primary* stress response comprises two major neuroendocrine components that result in the release of catecholaminergic and corticosteroid stress hormones. The first component is a rapid activation of the sympathetic nervous system whose endpoint is the release of catecholamines into the blood from the chromaffin tissue in the head kidney. This is coupled with a slower endocrine cascade referred to as the hypothalamus-pituitary-interrenal (HPI) axis, resulting in the release of the corticosteroid cortisol into the blood stream (Ellis et al., 2012; Pottinger, 2008).

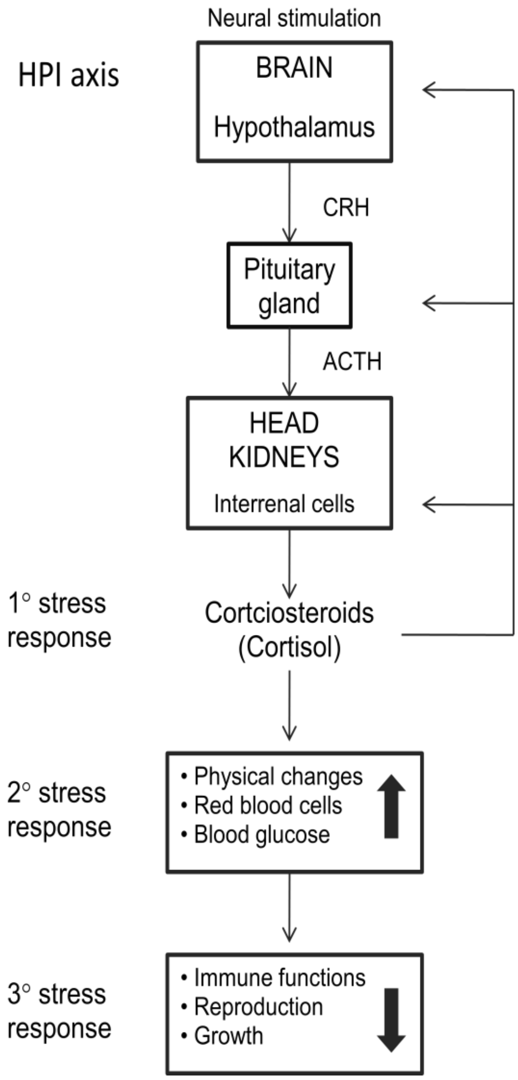

Figure 1. Simplified diagram of the stress response in fish, including the hypothalamus-pituitary-interrenal (HPI) axis (After Ellis et al., 2012 and Wendelaar Bonga, 1997). Corticotrophin releasing hormone (CRH) and adrenocorticotropin (ACTH). Arrows represent negative feedback mechanisms.

A simplified diagram of the processes of HPI axis is given in Figure 1 (After Wendelaar Bonga, 1997). To briefly describe the process, the HPI cascade is triggered in higher brain centres stimulating the hypothalamus to release corticotrophin releasing hormone (CRH). This in turn stimulates the pituitary gland to release adrenocorticotropin (ACTH) which triggers the subsequent synthesis and secretion of cortisol, the primary corticosteroid hormone, from the interrenal tissue in the head kidney (reviewed by Huntingford et al., 2006; Wendelaar Bonga, 1997).

The *secondary* stress response is triggered by the stress hormones released during the primary stress response. This results in physiological changes, such as an increased number of red blood cells, alterations of cardio-respiratory functions that increases respiratory capacity, heart rate and stroke volume, increased blood flow to the gills, mobilisation of energy sources such as carbohydrate and lipid reserves, depletion of glycogen stores, increased plasma glucose levels, high muscle activity, anaerobic glycolysis and an increase in plasma lactate (reviewed by Ellis et al., 2012; Huntingford et al., 2006). Furthermore, accompanying these physiological adjustments are alterations in behaviour, such as a reduced feeding activity (reviewed by (Ashley, 2007; Ellis et al., 2012; Huntingford et al., 2006; Pottinger, 2008).

The *tertiary* stress response may arise if the secondary stress responses are maintained over a prolonged period of time (Ellis et al., 2012). At this stage, continuous activation of the HPI axis (cortisol) can become maladaptive with deleterious consequences at the whole-animal level. Outcomes can include: reproductive dysfunction, immune-suppression resulting in increased mortality, susceptibility to disease and suppressed growth (reviewed by Ellis et al., 2012; Pottinger, 2008). Specifically for growth, many of the adaptive elements of the acute stress response affect energy intake and increase energy utilisation, resulting in reduced growth (Huntingford et al., 2006). This has been termed allostatic load, in animals, which is a chronic deviation of the regulatory system from its normal operating level (Korte et al., 2007; Wingfield, 2005). The elevated glucocorticoid concentrations that accompany repeated or prolonged stress put an energetic demand on the body (Goymann and Wingfield, 2004; Korte et al., 2005), resulting in trade-offs with other energy-demanding functions, such as growth, ultimately leading to maladaptive performance (Schreck, 2010; Segner et al., 2012). Ultimately, such changes significantly compromise the welfare of fish (Huntingford et al., 2006).

2.2.2. Indicators of stress

Various biochemical parameters at the three levels of the stress response have been used as potential indicators of stress and have been used to assess welfare in fish (reviewed by Ellis et al., 2012). Furthermore, other indicators of the neuroendocrine stress response, such as serotonergic activity (see below), have been used to assess social stress in fish (Winberg et al., 1991; Winberg et al., 1992; Winberg and Nilsson, 1993; Øverli et al., 1999), although only recently with reference to welfare (McKenzie et al., 2012).

The most widely used indicator of HPI activation in studies concerning stress in fish is blood levels of the stress hormone cortisol, as it provides the most meaningful measurement of stress in fish for several reasons. Most importantly, it provides an immediate measure of the secretory activity of the interrenal tissue and therefore provides a value that most closely equates to the concentration the target tissues are exposed to (Pottinger, 2008). Additional advantages of cortisol measures are that they are not prone to sampling effects, the sampling procedures is relatively straightforward and quick, sampling is not necessarily terminal, and cortisol analysis procedures have been standardised with the radio-immuno (RIA) and enzyme-linked immunosorbent (ELISA) assays. Furthermore, the cortisol response is typically strong for a variety of acute and chronic stressors. Moreover, it plays a regulatory role in many important physiological processes (reviewed by Ellis et al., 2012).

Generally, following an acute stressor, cortisol concentrations return to pre-stress levels within hours of exposure to the stressor (Pickering and Pottinger, 1989). In contrast, when exposed to a chronic stressor, elevated cortisol levels generally persist for longer periods of time, such as a period of several weeks (Pickering and Pottinger, 1989). However, in some cases, cortisol levels do not remain elevated, with levels returning to basal levels within a week (Pickering, 1992), representing a form of acclimation to the chronic stressor (Ellis et al., 2012; Pickering, 1992).

The neuroendocrine stress response can be used, such as the monoamine neurotransmitters serotonin (5-HT), dopamine (DA) and norepinephrine (NE) in the brain that influence the primary stress response (Øverli et al., 2001). In particular, central 5-HT plays an important role, integrating the behavioural and neuroendocrinal stress response (Winberg and Nilsson, 1993; Øverli et al., 2001). Serotonergic, quantified as the ratio of the immediate metabolite of 5-HT; 5-hydroxyindoleacetic acid, 5-HIAA and 5-HT is often correlated to plasma cortisol concentration, suggesting a stimulatory role of 5-HT activity on the HPI axis (Øverli et al., 1999). However, in cases where this relationship weakens, such as during prolonged stress where HPI axis reactivity (cortisol) decreases, 5-HT activity can remain high (Winberg and Lepage, 1998) and therefore provides an indicator of chronic stress. Indeed, previous studies investigating social behaviour in pairs or small groups of fish found have found an elevation in serotonergic activity levels in individuals exposed to prolonged periods of social stress (socially subordinate individuals) (Winberg et al., 1991; Winberg et al., 1992; Winberg and Nilsson, 1993; Øverli et al., 1999).

Besides measures of the neuroendocrine stress response, welfare measures associated with the tertiary effects of the stress response are used as direct and indirect indicators of chronic stress (Conte, 2004). These include measures of reductions in growth performance, suppressed reproductive function, diminished immune function and disease resistance (reviewed by Ashley et al., 2007).

Reductions in growth performance have been observed in individuals exposed to chronic stress. For example, Barton et al. (1987) and Gregory and Wood (1999) found that chronic elevation of cortisol, due to cortisol treatment, had a negative effect on growth rate in rainbow trout. A study by Jentoft et al. (2005) investigated the consequences of frequently stressed fish on growth performance and found that growth was reduced in these fish. Stress has also been linked to suppressed reproductive performance. It has been found that stress could affect certain reproductive performance parameters at the final stages of sexual maturation of female rainbow trout, such as the timing of ovulation and relative fecundity Contreras-Sanchez et al. (1998). With regards to diminished immune function, Einarsdottir et al. (2000) studied the effects of acute handling stress on the primary and secondary antibody responses in Atlantic salmon *Salmo salar* and found that severe stress at the time of immunisation was immune-suppressive in fish. Furthermore, there is some evidence that stressors can increase mortality in rainbow trout by increasing their susceptibility to disease (reviewed by Pickering et al., 1992). As an example, Pickering and Pottinger (1989) demonstrated a correlation between plasma cortisol concentrations and mortality rate due to disease in brown trout *Salmo trutta*.

2.3. Why is fish welfare important?

Fish welfare represents an important issue for the aquaculture industry from both a practical and ethical point of view. From a practical point of view, production efficiency, quality and quantity are often coupled with good welfare. Additionally, the public is increasingly concerned about the welfare of farmed fish, highlighting the ethical significance of fish welfare (Ashley, 2007). As a result, fish welfare has become a growing area of research (Ashley, 2007), in an attempt to develop husbandry techniques that promote welfare in farmed fish (Huntingford and Kadri, 2009). To illustrate this, a search using the keywords ‘welfare’ and ‘aquaculture’ as the topic in the Web of Science (WoS) reveals that there has been a sharp increase in the number of publications since the early 1990's (WoS, February 2013; Figure. 2).

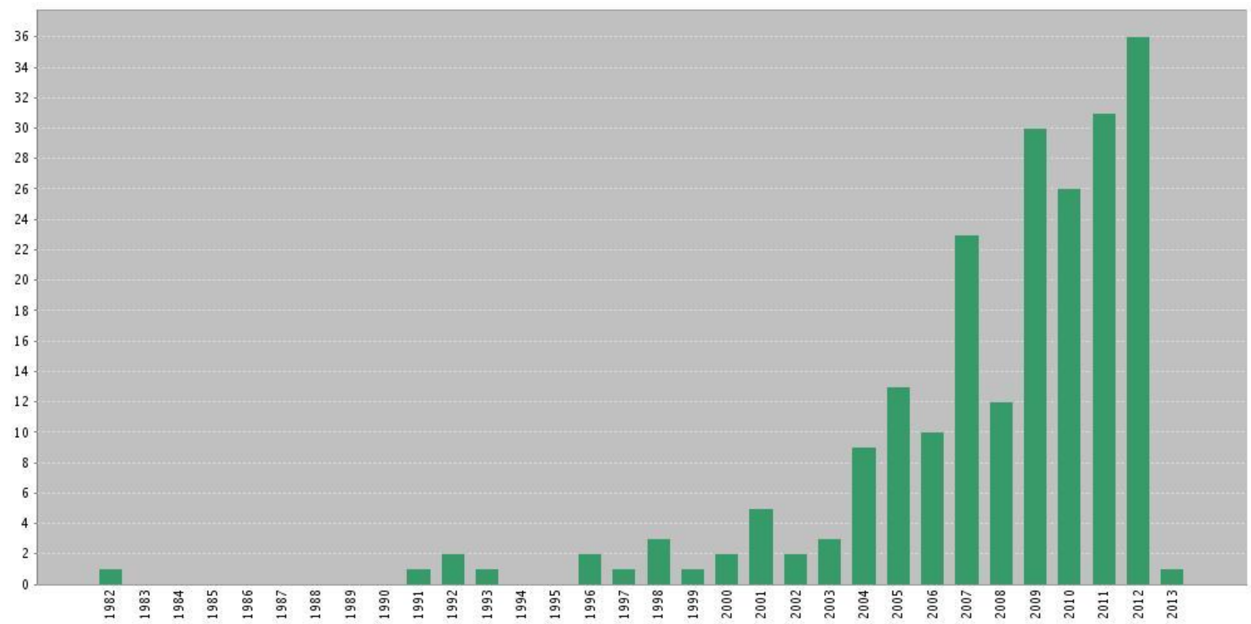

Figure 2a. The number of published studies on the topic 'welfare and aquaculture' published each year (From WoS, February 2013)

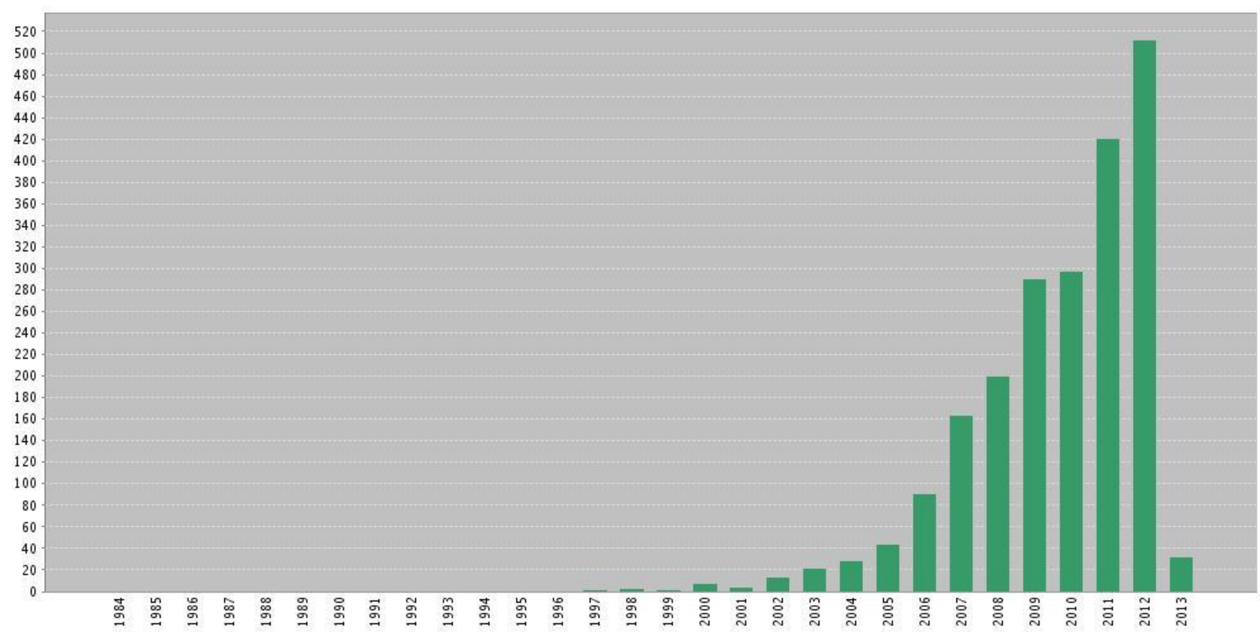

Figure 2b. The number of citations on the topic 'welfare and aquaculture' published each year (From WoS, February 2013)

In aquaculture, fish are exposed to a range of industry practices that may act as chronic stressors which potentially compromise welfare. The effects of a wide range of aquaculture practices on the stress physiology of fish are well documented, and have been reviewed by

Conte (2004) and Pickering (1992). Some of these practices include frequent handling, transport, periods of food deprivation, deteriorating water quality, and sub-optimal stocking densities and social environments (Ashley, 2007; Huntingford et al., 2006).

2.4. The relationship between stocking density and fish welfare

Rearing density is normally defined as the weight of fish per unit volume of water (Ellis et al., 2002) and typically refers to the concentration at which fish are initially stocked in a system. Furthermore, crowding is often loosely referred to high rearing density (Ashley, 2007; Huntingford et al., 2006).

Rearing density in the aquaculture industry has been highlighted as an area of particular concern with respect to welfare during recent years (Ashley, 2007; Ellis et al., 2002). Presumably, this is a consequence of increasing public concern about the welfare of fish in aquaculture (Huntingford et al., 2006), and recognition by the commercial and scientific communities that inappropriate densities can contribute to a reduced welfare status in fish (Ellis et al., 2002).

Rearing density encompasses a complex web of interacting factors, such as water quality, social interactions, fish to fish interaction and fish to housing interaction that can have an effect on many aspects of welfare (Ashley, 2007; Ellis et al., 2002; Turnbull et al., 2008). Furthermore, this relationship between rearing density and welfare is often variable between studies, attributed to the study specific nature of each study, indicating the specific environmental conditions act to influence how stocking density affects welfare (Ellis et al., 2002). As such, it is very difficult to make generalisations about how rearing density affects welfare for all situations (Turnbull et al., 2008).

There are a few limiting factors related to density and the capacity of the rearing system, especially in intensive culture. The biomass that a system can support is determined by the amount of oxygen that can be delivered to the fish and the waste removal capacity of the system. Inadequate amounts of oxygen delivery and waste removal can have negative consequences for welfare (Conte, 2004).

Furthermore, although there is still debate about which factor is the primary cause of negative welfare, it has been identified that the effects of rearing density on the welfare of farmed salmonids appears to be mediated through water quality and social interactions (reviewed by Ellis et al., 2002; Conte, 2004).

2.4.1. Water quality

Attention has been drawn to oxygen and ammonia as the water quality parameters generating the observed density effects (reviewed by Ellis et al., 2002). For example, increasing densities can reduce dissolved oxygen (DO) levels and increase un-ionised (UIA) concentrations in the water, depending on the pH (Ellis et al., 2002). As a result, low DO and high UIA levels, the latter being toxic to fish, can act as chronic stressors to rainbow trout, elevating plasma cortisol levels (Pickering et al., 1991). Person-Le Ruyet et al. (2008) found that growth rates were depressed when water quality was low and attributed this to low levels of oxygen and high UIA concentrations. Indeed, the threshold oxygen concentration for growth in rainbow trout has been shown to be ~ 75 % saturation (Pedersen, 1987). Moreover, low water quality (high UIA) has been correlated to the increased severity of fin erosion (North et al., 2006), although aggressive interactions have been highlighted as the primary cause (see below).

2.4.2. Behavioural interactions

Stocking density has a large effect on social interactions between fish. This is the passive non-aggressive behavioural interactions, such as collision and abrasion with conspecifics and the physical tank environment, as well as aggressive behavioural interactions between conspecifics that can be detrimental to welfare (reviewed by Ellis et al., 2002).

There is some evidence to suggest that increasing densities may cause physical injury due to abrasion with other fish as well as the rearing environment. For example, it has been found that tight grouping of trout at feeding times resulted in abrasion with conspecifics, removing the protective mucus layer (Winfree et al., 1998). Furthermore, abrasion with the rearing environment has been proposed as a cause of fin damage (Abbott and Dill, 1985) although no direct observational evidence exists for this interpretation (reviewed by (Ellis et al., 2008).

The aggressive behavioural interactions observed in Salmonids have been particularly highlighted in relation to welfare (Ellis et al., 2002). There is evidence for the formation of dominance hierarchies in rainbow trout held at low densities (North et al., 2006). This has also been demonstrated in small experimental populations of rainbow trout where the formation of dominance hierarchies is frequently observed (Noakes and Leatherland, 1977). It is commonly assumed that in salmonids, aggression decreases with increasing density. This may because establishing and maintaining ordered dominance hierarchies becomes increasingly difficult at high densities, thereby decreasing the quantity of aggressive acts (Alänärä and Brännäs, 1996; Bagley et al., 1994; Li and Brocksen, 1977). Therefore, by increasing rearing density, the damaging territorial aggressive behaviour can be altered to schooling behaviour in salmonids (Grand and Dill, 1999). For example, there is evidence that rainbow trout show shoaling behaviour in intensive culture, which has been shown to reduce aggressive behaviour (reviewed by Ellis et al., 2002).

There are several detrimental effects of aggressive behaviour on aspects of welfare such as fin erosion, body injury, social stress, loss of appetite, suppressed growth rate, elevated metabolic rates, and disease and mortality (reviewed by Ellis et al., 2002):

There is evidence that aggressive behaviour in small groups of rainbow trout can cause fin and body injury. Abbott and Dill (1985) observed the frequency of aggressive nips in rainbow trout, associated with fighting and establishment of dominance hierarchies, to be related to fin damage. Furthermore, the authors observed attacks directed at the body resulting in scale loss. They also suggested that that these aggressive attacks could cause damage to the gills, eyes and mouth (Abbott and Dill, 1985). Additionally, increased external damage to scales and fins in combination with reduced immune-competence (Olsen and Ringø, 1999) has been attributed as a cause for increased susceptibility to infectious diseases (Pottinger and Pickering, 1992). Social interaction within small groups of salmonids can cause differing levels of social stress among individuals. Subordinate individuals have been found to show various physiological evidences of being in a stressful situation including raised plasma cortisol levels, while dominants tend to show indications of not being stressed (Pottinger and Pickering, 1992). Socially induced stress is commonly thought to reduce appetite and foraging behaviour or direct competition for feed, resulting in a reduced feed intake and thereby reduced growth rates in subordinates (Gregory and Wood, 1999; Jobling, 1985). A less efficient feed conversion has also been recorded in socially stressed subordinate fish, perhaps due to metabolic changes resulting from differing levels of social stress (Abbott and Dill, 1989; Li and Brocksen, 1977). Besides chronic social stress, the high activity levels associated with active aggressive interactions may be energetically costly, resulting in greater energy expenditure and metabolic rates, and consequently decreased feed utilisation and growth (Li and Brocksen, 1977). Furthermore, a lower rate of dietary nutrient utlisation in subordinates may influence growth rates (Olsen and Ringø, 1999).

2.5. State of the art: stocking density and welfare of farmed rainbow trout

There is a considerable amount of literature that has investigated the relationship between stocking density and indicators of welfare in farmed rainbow trout *Oncorhynchus mykiss* that has been reviewed by Ellis et al. (2002). Although in the majority of the studies did not make reference to welfare *per se*, the studies investigated the effects of varying density levels on indicators of welfare such as, performance (e.g. mortality, feed intake, feed conversion ratio, body condition index, growth), condition (e.g. fin and gill condition), and stress levels (e.g. plasma cortisol, plasma glucose, and haematocrit) The authors concluded that despite the lack of clear evidence, high stocking density had the potential to reduce welfare. For example, increased fin erosion and gill damage, reduced food intake, food conversion efficiency, nutritional condition, growth rate and immune function have been demonstrated at high densities. However, a too low density was also concluded to be detrimental to welfare, as it was found to result in reduced feed intake and increased mortality, as a result of extreme aggressive behaviour between conspecifics (reviewed by Ellis et al., 2002).

2.5.1. Recent findings on effects of density on welfare and growth

Since then, additional studies have been carried out, investigating the relationship between stocking density and rearing or environmental conditions on different aspects of growth performance and welfare in rainbow trout:

For example, Boujard et al. (2002) investigated the effect fish held at stocking densities of 25, 70 and 100 kg m^{-3} submitted to different levels of food accessibility on feed intake, feed utilisation and feeding behaviour. The authors concluded that reduced feed intake and resulting decrease in growth at high density was due to food accessibility, and not crowding stress (Boujard et al., 2002).

North et al. (2006) studied the impact of the stocking densities of 10, 40 and 80 kg m^{-3} on a variety of physiological and morphometric indicators. They demonstrated that being held at high density (80 kg m^{-3}) did not have consistent effects on growth rates or physiological indicators of welfare, despite increased fin erosion. Furthermore, they found evidence for stronger dominance hierarchies at low density (10 kg m^{-3}). Consequently, it was concluded that both low and high stocking densities had the potential to compromise welfare (North et al., 2006).

A study by Rasmussen et al. (2007) examined the influence of the combined effects of stocking density, fish size and feeding frequency on fin condition and indicators of growth performance in two experiments. The first experiment showed that there was no effect of density (41 and 92 kg m^{-3}) on indicators of growth performance. However, an effect of density and fish size (70 or 125 g) acted together to impair fin condition. The second experiment showed that growth performance was reduced at high density (124 kg m^{-3}) compared to low density (45 kg m^{-3}), but that fin condition was improved at high density (Skøtt Rasmussen et al., 2007).

The combined effects of stocking density (25, 74 and 120 kg m^{-3}) and water quality (low and high) on indicators of welfare and growth were studied by Person-Le Ruyet et al. (2008). They concluded that growth performance was best under high water quality conditions at all densities and that, irrespective of water quality, growth performance was the worst at the high density (120 kg m^{-3}) despite not observing any major physiological disturbances (Person-Le Ruyet et al., 2008).

There are two studies that have investigated the combined effects of stocking density (~ 25 and ~ 100 kg m^{-3}) and sustained exercise (water current of 0.9 bl s^{-1}). The first study showed that high density, irrespective of water current, resulted in a lower growth performance. Furthermore, water current was shown to have a positive effect on energetic budgets, reducing metabolic rate irrespective of density, and was attributed to induce schooling behaviour thereby reducing aggressive behaviour and stress (Larsen et al., 2012). The second study showed that growth rates were reduced at high density, irrespective of water current, and this was attributed to high energy used. The authors concluded that this was unlikely to be due to chronic stress, as cortisol values were low at all densities, but may have been due to an alteration in physiological state (McKenzie et al., 2012).

The overall picture arising from the studies performed to date investigating the effects of stocking density on different parameters suggests that both low and high densities are potentially detrimental to welfare in rainbow trout. Interestingly, what is considered low density and what is considered high density appears to be quite ambiguous, as these ‘definitions’ vary between studies. Furthermore, the results of these studies clearly illustrate the complex nature of the interaction between stocking density and fish welfare, with several environmental factors interacting together and with density to influence indicators of welfare and performance. As a consequence, it is also a complex undertaking to model these multiple interacting and confounding influences of stocking density on measures of welfare (Turnbull et al., 2008), in an effort to gain an overall understanding.

2.5.2. Recommendations for stocking

A number of the reviewed studies have attempted to make specific recommendations for maximum rearing densities for rainbow trout, based on their experimental results (reviewed by Ellis et al., 2002). Depending on the type of rearing system, the recommendations ranged from 4 to more than 267 kg m^{-3} (Ellis et al., 2002). Clearly, wide discrepancies exist with regard to the maximum stocking density recommendations for rainbow trout (North et al., 2006) and the recommendations for optimal stocking from a welfare perspective remain elusive.

Such a wide range of recommendations highlights the fact that it has been a challenge to identify density limits that promote optimal welfare and production in rainbow trout. This is in part due to a lack of understanding of how the different environmental factors interact with each other and with stocking density to affect welfare (Ashley, 2007). Another reason is that the effect of density measures on welfare may vary greatly between studies due to the study-specific nature of experiments (Bagley et al., 1994; Holm et al., 1990; Procarione et al., 1999). For example, studies vary in experimental duration, water quality, density levels used, feeding method, size of the fish, life history of the fish, level of domestication, type of rearing system used and environmental conditions. A density threshold for one set of conditions may, therefore, not be relevant for another (Ashley, 2007) and makes comparison of the results between studies difficult. Some of the most standard study conditions from a few studies are given in Table 2, to illustrate differences in study specific conditions.

Table 2. Some of the most standard study specific conditions from a few studies investigating the influences of stocking density.

	Density (kg m^{-3})					
Study	Low	Int.	High	Size (g)	Temp. (°C)	Feeding
Boujard et al. (2002)	25	70	100	26.0 ± 1.5	~ 15.5	4 g 100g $^{-1}$ fish using belt feeders
North et al. (2006)	10	40	80	180 ± 35	Ambient	Hand fed in accordance with manufacturer's tables
Rasmussen et al. (2007)	41		92	70 and 125	17.7	Restricted feeding (2.1 % and 2.8 % per day for large and small fish respectively)
	45		124	125	16.4	Restricted feeding once or 3 times daily (2.0 % per day) Using belt feeders
Person-Le Ruyet et al. (2008)	25	74	120	111.5 ± 2.4		Computer controlled demand-feeders using reference reward level of the farm
Larsen et al., 2012	25		100	~160	19	1.5% of estimated body mass daily using belt feeders
McKenzie et al., 2012	25		100	~ 110	14	Fed to satiation using belt feeders

2.5.3. Alternative methods for identifying optimal density limits

The above discussion draws attention to the significance of developing and applying alternative methods to provide novel insight into the potential stocking density limits that are optimal for welfare in farmed rainbow trout.

Behavioural techniques have been suggested to provide promising tools for identifying conditions that promote good welfare in fish. Behaviour has numerous advantages in welfare studies as it is the result of an animal's own decision making processes (Dawkins, 2004). In other words, instead of deciding what is best for an animal, one could allow an animal to 'show' what is best for them through their behaviour.

Dawkins (2004) proposed the idea that animal welfare could be determined by answering two questions: firstly, if the animals are healthy and secondly, if the animals have what they want. The author suggested that behavioural techniques could be used to tell us about what animals need. Furthermore, one suggested technique was to use quantitative observations of the spatial distribution of animals (Dawkins, 2004). The spatial distribution that animals adopt in the rearing environment may provide valuable indicator of how they respond to each other, and what they need in terms of space and stocking density (Dawkins, 2004; Turnbull et al., 2008).

To our knowledge, this type of approach has not been investigated before in fish, and therefore provides a novel approach for investigating what fish may 'need' in terms of rearing density.

3. Aims of the PhD project

The overall objective of my PhD project was to investigate welfare aspects of stocking density in farmed rainbow trout *Oncorhynchus mykiss* using behavioural and physiological methods. Specifically;

3.1. To establish and apply a novel behavioural method for determining a level of crowding experienced as aversive by the fish using spatial distribution in two-tank systems (Paper I);

3.2. To study the influence of these levels of crowding on indicators of welfare as assessed by: oxygen consumption, scale loss, fin erosion, and neuroendocrine stress levels (Paper II);

3.3. To study the effect of these levels of crowding on indicators of growth performance and measures of energetics (Paper III).

4. Experimental work

The experimental work and methodology carried out during this PhD project has been described in detail in the Papers (see Appendices) and is therefore only summarised here, describing any additional details.

4.1. Experimental facilities

4.1.1. "Two-tank" facility

The two-tank facility was specifically designed and built at DTU Aqua for carrying out Experiment 1 (see section 4.2.1.). The facility consists of three replicates of a two-tank system, standing parallel to each other (Figure 3).

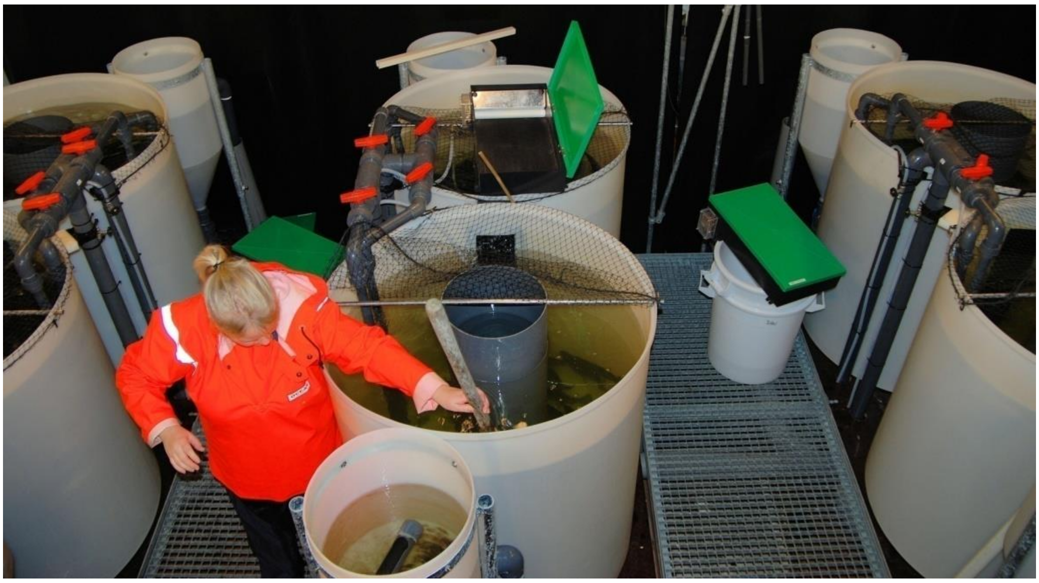

Figure 3: Picture of a two-tank facility. Picture by Peter V. Skov

Each two-tank system is made up of two white circular tanks attached to one another via a closable doorway (Figure 4), allowing the fish to move freely between the two environments. Each tank represents a separate environment, individually equipped with water inflow and outflow, with the option of creating water current, and an oxygen supply. The maximum capacity of each tank is approximately 700 liters, and there is the possibility for altering the water volume in each tank. The entire facility is run on the same recirculation biofilter system.

Figure 4: Picture of a two-tank system, as viewed from above. Picture by Peter V. Skov

4.1.2. "Physiology" facility

The "Physiology" facility at DTU Aqua (Figure 5) was used to carry out Experiment 2 (see section 4.2.2.). The facility consisted of 12 tanks that were identical to the ones at the "two-tank" facility, providing an adequate number of tanks for the experiment. Furthermore, the facility had been used previously for welfare studies in rainbow trout at DTU Aqua. Additionally, the tanks of the facility had been modified to function as respirometers, which provided the opportunity to do oxygen consumption measurements.

Figure 5: Picture of the physiology facility. Picture by Caroline Laursen

4.1. Experimental fish

4.1.1. Rainbow trout

Rainbow trout *Oncorhynchus mykiss* (Walbaum 1972) is a species of freshwater Salmonid farmed primarily in Europe and North America (Ellis et al., 2002). In Denmark, it is the most dominantly cultured species, with an annual production of about 31,000 tons in freshwater and about 9,000 tons in saltwater (Jokumsen and Svendsen, 2010).

The rainbow trout is a robust species, as they are fast growing and tolerant to a wide range of environments and handling (FAO, 2013). They are more tolerant to high densities that any other species of trout and salmon, which is thought to be due to a greater physiological tolerance to “crowding stress” (Ellis et al., 2002). Furthermore, there is an extensive literature on their life history and behaviour, and a range of studies have already been carried out in the field of welfare. Therefore, they were considered to be ideal experimental subjects for the present PhD project.

The rainbow trout used for the experiments in this PhD project came from local fish farms (see Materials and Methods section 2.1 in Paper I & II).

4.2. Experimental design

4.2.1. Experiment 1 (Paper I)

The design of the experiment was that gradually increasing the total stocking density in the two-tank system would result in a threshold for crowding in the “crowded” tank of the system. Accordingly, three stocking density levels were used during the experiment. One two-tank system was stocked at a total density of 20 kg m^{-3}, the second at 40 kg m^{-3} and the third at 80 kg m^{-3}. This gave the fish the opportunity to choose a density between the range of 20 and 160 kg m^{-3}. The number of fish in each tank was counted every three days during the experiment for a period of two weeks, to determine the spatial distribution of the fish in the two-tank systems. For practical reasons, this was done by counting the number of individuals in the “dominant” tank. By subtracting this count from the total count of fish in the two-tank system, it was possible to determine the number of individuals occupying the “crowded” tank. The percentage of fish in the “crowded” tank was used as a measure of the spatial distribution pattern between the two tanks of each system.

As we observed that the distribution of the fish changed between the night time and the day time, the fish count was determined at two time points during the daily cycle. One time point was chosen at the end of the night time hours, which was in the morning at 07:30 when it was still dark. The second time point was chosen at the end of the day time hours, which was in the evening at 19:30 when it was still light.

Additionally, a subsample consisting of six individuals from the "crowded" tank was sampled for blood and brain parts, to be analysed for plasma cortisol (see section 4.2.2.1) and brain serotonergic activity levels (see section 4.2.2.2.) to determine stress levels. The subsample was taken before the number of fish in the tank was determined, so as not to disturb the fish and influence the stress levels. Determining the stress levels of the individuals occupying the "crowded" tank would provide us with an indication of crowding stress, and thereby indicate a level of aversion to the level of crowding in the tank.

4.2.2. Experiment 2 (Paper II, III & unpublished data)

The densities used in experiment 2 were based on the results obtained from experiment 1. Three densities were investigated; a density of 25 kg m^{-3} served as an un-crowded low density (LD), the highest density accepted by the fish without showing indications of crowding stress (in experiment 1) of 80 kg m^{-3} as the intermediate density (ID), and the highest density accepted by the fish showing indications of crowding stress (in experiment 1) of 140 kg m^{-3} as the high density (HD). The results from experiment 1 indicated that the average absolute density in one tank of the two-tank system ("crowded" tank) was approximately 126 kg m^{-3}. However, in the "light", this density was higher, and was at 137 ± 10.0 kg m^{-3}. We therefore decided to round up this value to 140 kg m^{-3} as the highest density. For the intermediate density, the results from experiment 1 indicated that the average absolute density in one tank of the two-tank system was approximately 69.9 ± 3.3 kg m^{-3} In the "light", this value was at approximately, and rounding up this value to 80 kg m^{-3}.

The fish were stocked randomly into the experimental tanks; at 25 kg m^{-3}, 80 kg m^{-3} and 140 kg m^{-3} in triplicate. A subsample of 30 individuals from each tank was pit tagged and adipose fin clipped for individual identification throughout the experiment. The 30 fish were added to the tank and the remaining biomass was added to each tank to acquire the desired density.

After stocking, the fish were acclimated for a period of approximately two weeks where the feeding level was gradually increased to 1.5% of their biomass per day. At the end of the acclimation period, the biomass in each tank was determined and re-adjusted to the desired density by removing excess kilograms. The number of fish in the tank was counted.

The experimental duration was 28 days, divided into two growth periods of 12 days. During each growth period, the fish were fed for 12 days, followed by a day of starvation and a day of weighing. Each tank was fed at 1.5 % of the estimated tank biomass per day, with 3 mm pellets (EFICO Enviro 920, BioMar A/S). The fish were fed in the morning at 09:00 with automatic belt feeders for a period of six hours. In the afternoon after feeding, the solid waste was collected from the whirl separator and the numbers of uneaten pellets were counted to determine feed waste. During pellet collection, the scales lost by the fish were also separated and collected for later weighing. After 12 days of feeding, the fish were starved for a day and weighed the following day.

On the day of starvation, to determine basal stress levels, four individuals from each tank (un-pit tagged) were sacrificed and blood and brain samples were collected from these fish. The blood samples were for later plasma cortisol concentration analysis and the brain samples for later brain monoamine and metabolites analysis.

On the day of weighing, the total biomass in each tank was recorded. Each tank was re-stocked with biomass to achieve the desired density and the excess fish were discarded. Furthermore, the sub samples of fish from each tank were individually weighed, measured for fork length and checked for fin damage at each subsequent weighing session.

Oxygen consumption was measured continuously throughout the growth period. Measurements were started on the first day of feeding and stopped on the day of weighing. Oxygen levels were set at 80% in the tanks held at the low density, 110% at the intermediate density, and 120% at the high density. Oxygen concentrations were set at these levels for practical reasons, to obtain a long enough closing period to be able to measure oxygen consumption and to ensure that oxygen levels did not fall below the critical level (60%) at the intermediate and high densities during the oxygen consumption measurement period. It has previously been shown that growth is not affected by oxygen levels above 75% (Pedersen, 1977).

Water quality parameters; NO_2, NO_3, pH, and temperature, were measured daily at the system level to ensure that they were within optimal levels for the fish. The temperature of the water in the system was controlled at 16 °C. A slow water current of approximately 0.5 body lengths per second was provided to each tank to even out the distribution of the fish and the pellets in the tank. Light conditions were at 14.5 light and 9.5 dark hours, with the lights automatically switching on at 07:30 and switching off at 22:00.

At the end of the experiment, an acute stress test was done. Following the final weighing session for the second growth period, the fish were left to acclimate for a period of a week. Each tank in succession, the water level in the tank was lowered for a period of one hour, after which the water level was returned to normal levels. Blood and brain samples were taken periodically after the acute stressor from a subsample of four individuals (see section 4.3.2.8.).

4.3. Experimental methods

4.3.1. Behavioural methods (Paper I)

4.3.1.1. Spatial distribution of fish in two-tank systems

The original approach was to apply the concept of the “Ideal Free Distribution” (IFD) model in the two-tank system to determine levels of crowding accepted by the fish. According to this concept, individuals are distributed between environments in proportion to the amount of resources that are available there ((Fretwell, 1972)). On this line, it was speculated that if the resource was increased in one environment there would be an increase in the amount of individuals there, and vice versa if the resource was decreased in one environment there would be a decrease in the amount of individuals there. Based on this reasoning, the design of the experiment was to gradually decrease the feeding level in one tank of the two-tank system, while gradually increasing the feeding level in the second tank of the system and allowing the fish to distribute themselves freely between the two tanks accordingly. The density obtained, when the relation between differences in feeding ratio and fish density no longer existed, would have been used as an indicator of crowding stress.

During pilot studies it was observed that when groups of fish were placed in a two-tank system, the result was an unequal distribution of individuals between the two tanks (Figure 6), despite the equal quantity of feed given in each tank. The resulting distribution pattern typically observed was one tank becoming occupied by a few dominant aggressive individuals and the majority of the fish occupying the second tank. The few dominant individuals occupying one tank drove out the majority of the group into the second tank, thereby controlling the distribution of the group in the two-tank system. This distribution pattern resembled an “Ideal Despotic Distribution” (IDD), first described in birds, where movement between patches was controlled by intraspecific competition (Fretwell, 1972). The IDD has also previously been described in laboratory situations in Salmonids, where dominant individuals excluded other individuals from a favourable patch (Hakoyama and Iguchi, 2001; Maclean et al., 2005). In our study, although behavioural quantifications of the individuals in the “dominant” tank were not carried out, observation of the fish confirmed that they displayed behaviour that was characteristic for a dominant individual. They displayed territorial behaviour, monopolising the food resource with chasing out individuals entering the tank. Furthermore, if more than one individual was present they displayed agonistic behaviour towards each other. The tank occupied by the dominant individuals will be referred to as the “dominant” tank and the tank holding the majority of the fish as the “crowded” tank.

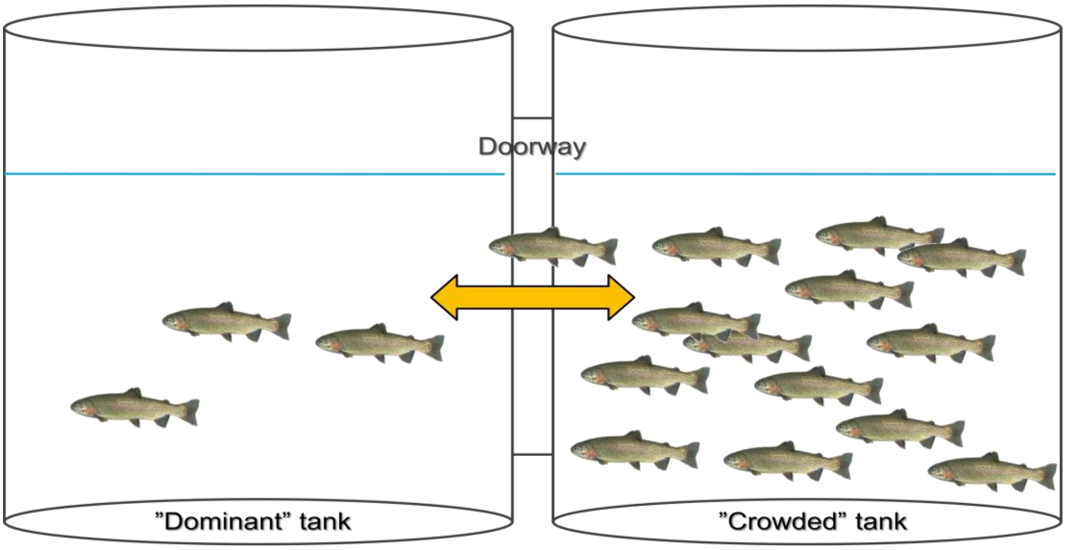

Figure 6. Diagram of the spatial distribution of groups of fish in a two-tank system

As a result of these observations during the pilot studies, it was evident that using the concept of IDF was going to be un-successful. Therefore, we decided to use the inequality in the distribution pattern to our advantage. The spatial distribution of the fish in the two-tank systems was used to investigate a level of crowding that the fish experienced as aversive (see section 4.2.1.).

4.3.2. Physiological methods (Paper I, II, III & unpublished data)

4.3.2.1. Plasma cortisol

During blood sampling in Experiment 1 and Experiment 2, the subsample of individuals taken during the first experiment (Paper I) and the second experiment (Paper II) were sacrificed by an overdose of anaesthetic (Ethylene glycol monophenyl ether). The blood samples were collected from the caudal vein of each individual using 1 ml syringes. Each syringe contained a small amount of EDTA, to prevent the blood from coagulating. The blood samples were transferred from the syringes to 1 ml eppendorf tubes and centrifuged at 15,000 rpm for 5 minutes. After centrifuging, the plasma was separated out into 1 ml eppendorf tubes and frozen at -80 °C for later analysis.

Plasma cortisol concentrations were quantified using the ELISA kit standard method (Neogen, Product #402710) (Paper I & II). Plasma cortisol concentrations from the acute stress test were quantified using Radioimmunoassay (unpublished data).

4.3.2.2. Brain serotonergic (5HIAA/5HT) activity

During brain sampling in Experiment 1 and Experiment 2, brain samples were taken from the same subsample of individuals as for blood sampling. Following blood sampling, whole brains were dissected out from each fish and separated into four parts; the brain stem, hypothalamus, telencephalon and optic lobes. Each brain part was frozen immediately in liquid nitrogen and stored separately at -80 °C for later analysis.

Before analysis, each frozen brain part was individually weighed. After weighing, the brain part was homogenised in a homogenising reagent (4% perchloric acid, 0.2% Ethylenediaminetetraacetic acid, 40 ng ml $^{-1}$ dihydroxi benzylamine hydroxide solution). The solvent was then centrifuged at 10,000 rpm at 4 °C for 10 minutes, separated into a 1 ml eppendorf and re-frozen at -80 °C.

The supernatant was assayed by High Performance Liquid Chromatography (HPLC) with electrochemical detection to quantify the concentration of 5-HT (serotonin) and its catabolite 5-Hydroxyindoleacetic acid (5-HIAA).

4.3.2.3. Oxygen consumption

Oxygen consumption measurements were taken continuously using the automated respirometry system. Every hour, the three-way valve at the inflow to the tank would close automatically and shut off the oxygen supply to each tank. The valve remained closed for a period of 6 minutes during the day time hours (09:00 – 17:00) and 8 minutes during the night times hours (18:00 – 08:00). It was done this way, as the fish used the oxygen more quickly during the day time hours, so the closing off period had to be shortened. The decline in oxygen concentrations in the tanks were registered from the transmitters to the data logger every 20 seconds during the period when the valve was closed.

Oxygen consumption was calculated as previously described in detail by McKenzie et al. (2012) Larsen et al. (2012). Briefly, oxygen consumption rates were calculated from the slope value obtained from the decline in oxygen in the tank, the estimated total biomass of fish in the tank on the day (using the SGR) and the total volume of water in the tank on the day. To ensure direct comparability of the concentrations of oxygen consumed between the tanks, the data used was selected from the days where the body mass increase of the fish was similar in each of the tanks. In the present study, a period of days from when the fish grew from 190 to 220 grams (mean body weight 205 grams) was chosen. The data on the amount of oxygen consumed taken from those days was corrected to a 205 gram body weight fish, using the method detailed by Larsen et al. 2012.

4.3.2.4. Fin erosion

Fin erosion was determined from the subsample of 30 pit tagged individuals from each tank, using the photographic key developed by Hoyle et al. (2006). During each weighing session, the individuals were separated from the tank biomass, lightly anaesthetized and examined. Each fin type per individual was compared to the pre-developed photographic key and given a score from one to five. A score of one was considered to be a fin in good condition and a score of five a fin showing considerable damage.

4.3.2.5. Scale loss

Fish scales were collected from each of the tanks daily. After feeding was finished in the afternoon, the solid waste from the tank that had collected in the whirl separator was flushed out and collected in a bucket. The contents of the bucket were emptied into a sieve where the scales were separated from the faeces and uneaten pellets. The scales were collected in plastic containers for later weighing.

The scales were dried and weighed on a pre-weighed filter paper (Qualitative filter paper, 413; WWR). Before weighing, the filter paper with the scales was put in the dryer at 60 °C for a period of one hour. To determine the grams of scales lost per kilogram fish (g kg^{-1}), the weight of the scales was divided by the estimated biomass in the tank (using the SGR). The total amount of scale loss was determined for each density treatments for the same period as for the total oxygen consumed, the selected days from when the fish grew from 190 grams to 220 grams (see section 4.3.2.3.).

4.3.2.6. Growth performance

The specific growth rate (SGR; % bw d^{-1}) was estimated for each tank from the total tank biomass (kg) between two time points using the equation: SGR = 100 * (Ln(W_f) - Ln(W_i)) / time. W_f was the final total biomass in the tank at the end of each growth period, W_i was the total initial biomass in the tank at the start of the growth period, and time (days) was the duration of the growth period.

The feed conversion ratio (FCR; kg kg^{-1}) was calculated for each tank using the equation: (feed intake / biomass gain). Ingested food for the period of investigation was estimated by subtracting the amount of feed waste from the amount of feed given per tank, and biomass fain was $W_i – W_f$.

4.3.2.7. Energetics

Energetic parameters were estimated based on the method described in detail by McKenzie et al. (2012) and Larsen et al. (2012). Using the specific growth rate (SGR), the biomass gain per day was calculated for each tank for the entire experiment. Based on these growth curves, a time period (days) was chosen for when the individual biomass gain was similar in all tanks. In the present study, the days from when the fish grew from 190 to 220 grams was chosen. The daily feed intake (kg) for this time period, the daily oxygen consumption rate (mg O_2 kg d^{-1}) for this time period was, the energy content (Kj g^{-1}) of the feed given (see section 2.3) and an oxycalorific coefficient (Kj mg^{-1} O_2) were used to calculate the energetic parameters for each tank.

4.3.2.8. Acute stress test

Assessing chronic stress in fish presents more challenges than assessing acute stress (Santos et al., 2010). Some studies have found that cortisol was not elevated in chronically stressed fish at high density (Pickering and Stewart, 1984; Procarione et al., 1999). This could be due to the fact that high rearing density is not a chronic stressor in rainbow trout, or alternatively that in fact it is a chronic stressor, but that their stress response had acclimated to the chronic stress at high density, due to a negative feedback mechanisms of cortisol that causes a down regulation of the hypothalamic–pituitary–interrenal (HPI) axis (Pickering and Stewart, 1984; Procarione et al., 1999; Santos et al., 2010). One way to test this is to assess the responsiveness of the HPI axis of fish held at different densities and examining the plasma cortisol response to an acute stressor (Ellis et al., 2012; Procarione et al., 1999; Santos et al., 2010)).

Here, an acute stress test was applied to each tank by reducing the water volume in the tanks for a period of one hour. In order to kept fish in the same crowding condition at 500 kg m^{-3}, the water volume was differently reduced in relation to stocking density treatments. After maintaining crowding for one hour, the water volume in the tanks was increased to original conditions and the fish were allowed to recover. Blood and brain samples were taken from each tank one hour before the acute stressor and then at time 0, 1, 2, 4, 8, and 22 hours following the acute stressor. A subsample of four individuals was sacrificed per sampling time point. Blood samples were taken using the same method described in section 4.3.2.1.

5. **Discussion of findings**

The following section provides a discussion about the main findings of the PhD project. Firstly, the results from Paper 1, using two-tank systems to investigate a density limit are discussed. Thereafter, the results from the indicators of welfare and performance of being held at this density limit, from Paper II, III and unpublished data, are discussed.

5.1. Spatial distribution in two-tank systems

The spatial distribution of the fish in the two-tank systems was established by counting the number of individuals occupying the “dominant” tank and subtracting this from the total count to determine the number of fish in the “crowded” tank. The percentage of fish in the “crowded” tank was used as a measure of crowding. Combined with the neuroendocrine measures of stress in this tank (see below), this provided a measure of aversiveness to crowding. The results indicated a negative relationship between the percentage of fish in the “crowded” tank and the total density stocked, with the percentage of fish occupying the “crowded” tank decreasing with increasing total stocking density (20, 40 and 80 kg m^{-3}; Fig. 7). In other words, there was a lower percentage of fish occupying this tank with higher total stocking density.

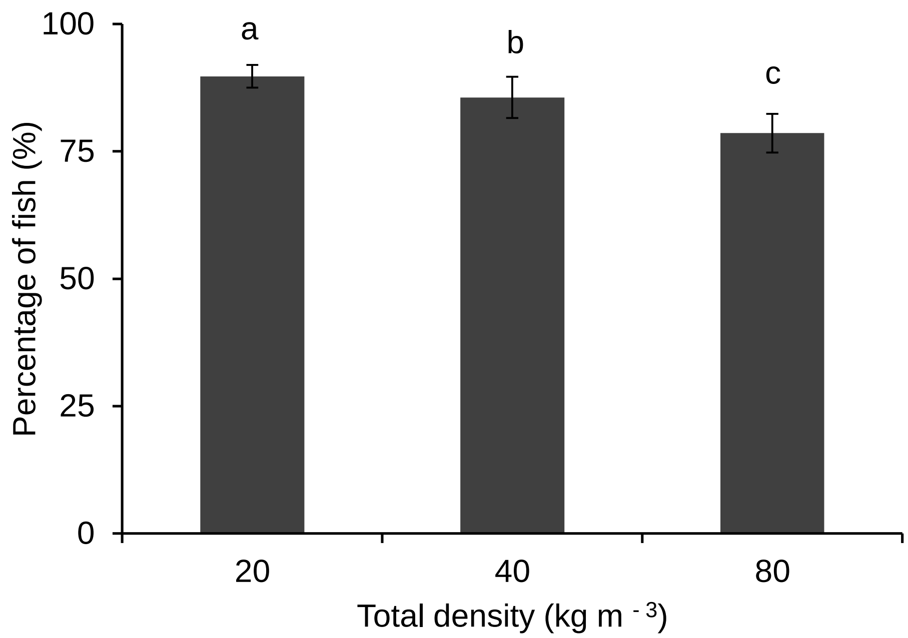

Figure 7. The percentage of fish in the “crowded” tank of the two-choice system between the three total densities (n=3). The letters (a, b & c) indicate a significant difference between treatments (From Paper I).

The distribution pattern observed between the two tanks was typically that the “dominant” tank was occupied by a few dominant aggressive individuals and the majority of the fish occupying the second “crowded” tank. However, it was observed that with increasing density, apart from the few dominant aggressive individuals occupying the “dominant” tank, there was a spillover of individuals from the “crowded” tank entering the “dominant” tank that did not perform aggressive acts. These individuals stayed immobile and accumulated in the “dominant” tank close to the doorway between the two tanks, a behaviour which is typically observed in subordinate fish. This distribution pattern was especially distinct in the two-tank system stocked with the highest total density, with the “dominant” tank occupied by a few dominant aggressive individuals and a gradual accumulation of subordinate individuals. This was likely a response to crowded conditions in the “crowded” tank at the highest density. In other words, when the aversiveness to the crowded conditions in the “crowded” tank became greater than being exposed to aggressive social interactions in the “dominant” tank, individuals from the “crowded” tank started moving over to the “dominant” tank, indicating that a level of aversiveness to crowding had been reached.

Neuroendocrine indicators of stress were examined to support our behavioural observations. They would provide an indication of stress levels in the “crowded” tank, and therefore reflect an aversion to the level of crowding in the tank. There was no difference between the total densities stocked (20, 40 and 80 kg m^{-3}) in the plasma cortisol concentrations in the “crowded” tank. In general, the plasma cortisol concentrations were low at all the total densities. However, there was a higher serotonergic activity level (5-HIAA/5-HT) in the brain stem of individuals in the “crowded” tank of the system stocked at 80 kg m^{-3} compared to the individuals in the two systems stocked at 20 and 40 kg m^{-3} (Fig. 8). The HPI-axis is suggested to be stimulated by serotonergic activity and often the two measures are found to be correlated during stress (Øverli et al., 1999). However, in some cases, this relationship can weaken with time, where reactivity of the HPI axis decreases while serotonergic activity remains high (Winberg and Lepage, 1998). Therefore, elevated levels of serotonergic activity and low concentrations of cortisol in the “crowded” tank of the two-tank system stocked at 80 kg m^{-3} were reflective of chronic stress in a crowded situation.

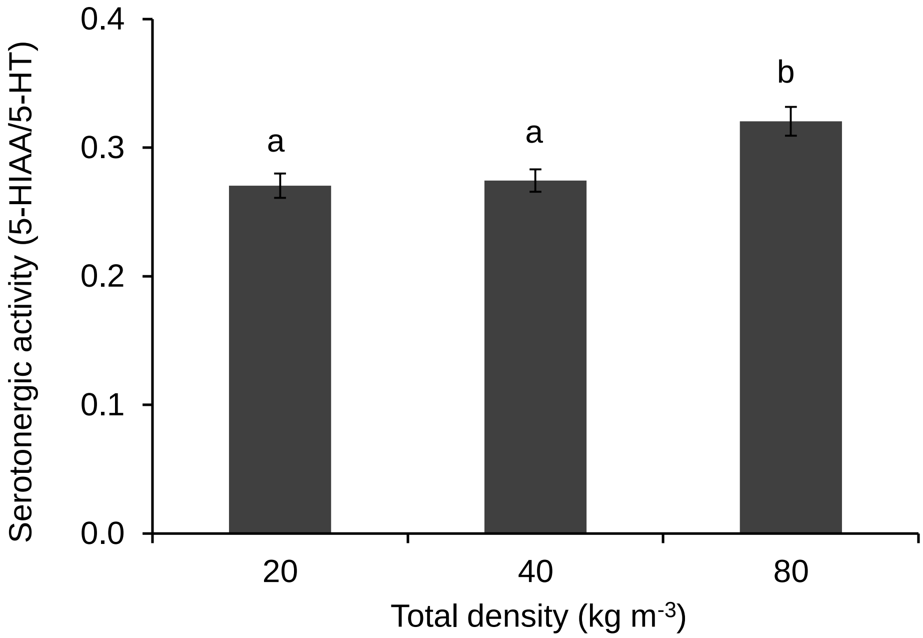

Figure 8. Serotonergic activity (5-HIAA/5-HT) in the Brain stem of individuals (n=18) in the "crowded" tank at each total density. The letters (a & b) indicate a significant difference between treatments.

The absolute density (kg m^{-3}) in the "crowded" tank of the two-tank systems was determined from the percentage of fish occupying this tank. At the highest density, where chronic stress due to a crowded situation was found, the absolute density, irrespective of the time of day, was 126.5 ± 3.7 kg m^{-3}.

Furthermore, although there was no significant difference in the percentage of fish occupying the "crowded" tank between the "dark" (morning) and "light" (evening), it was observed that the distribution pattern in the two-tank systems was different depending on the time of day. Interestingly, the serotonergic activity level in the brain stem of the individuals in the "crowded" tank was higher in the "light" than the "dark", irrespective of stocking density. The resulting absolute density in the tanks was therefore different depending on the time of day. In the "crowded" tank of the two-tank system stocked at the highest density, of 80 kg m^{-3}, the absolute density (kg m^{-3}) in the "dark" (morning) and "light" (evening) was 115.7 ± 5.5 kg m^{-3} and 137.4 ± 10.0 kg m^{-3} respectively.

5.2. Indicators of welfare and performance at revealed density limit

To assess welfare and performance of the fish held at the density level obtained in the study using two-tank systems (Paper I), oxygen consumption, fin erosion, scale loss, neuroendocrine indicators of stress, energetics and growth performance were used.

The minimum, median, maximum hourly oxygen consumption rates (mg O_2 kg $^{-1}$ hr $^{-1}$) and the total oxygen consumption (mg O_2 kg $^{-1}$) during the period when the fish in each tank experienced similar growth were used as measures of oxygen consumption. The results showed a negative relationship between oxygen consumption and stocking density. In other words, oxygen consumption decreased with increasing density. This pattern was also reflected in the daily cycle of oxygen consumption (mg O_2 kg $^{-1}$ hr $^{-1}$; Fig. 9).

The lower oxygen consumption rates at the density of 140 kg m^{-3}, compared to the density of 80 kg m^{-3} and 25 kg m^{-3}, could be behaviourally related and be indicative of reduced aggressive behaviour at this density. Indeed, high levels of spontaneous activity associated with agonistic behaviour have been shown to cause elevated metabolic rates in juvenile sockeye salmon and rainbow trout (Brett, 1964; Christiansen et al., 1991).

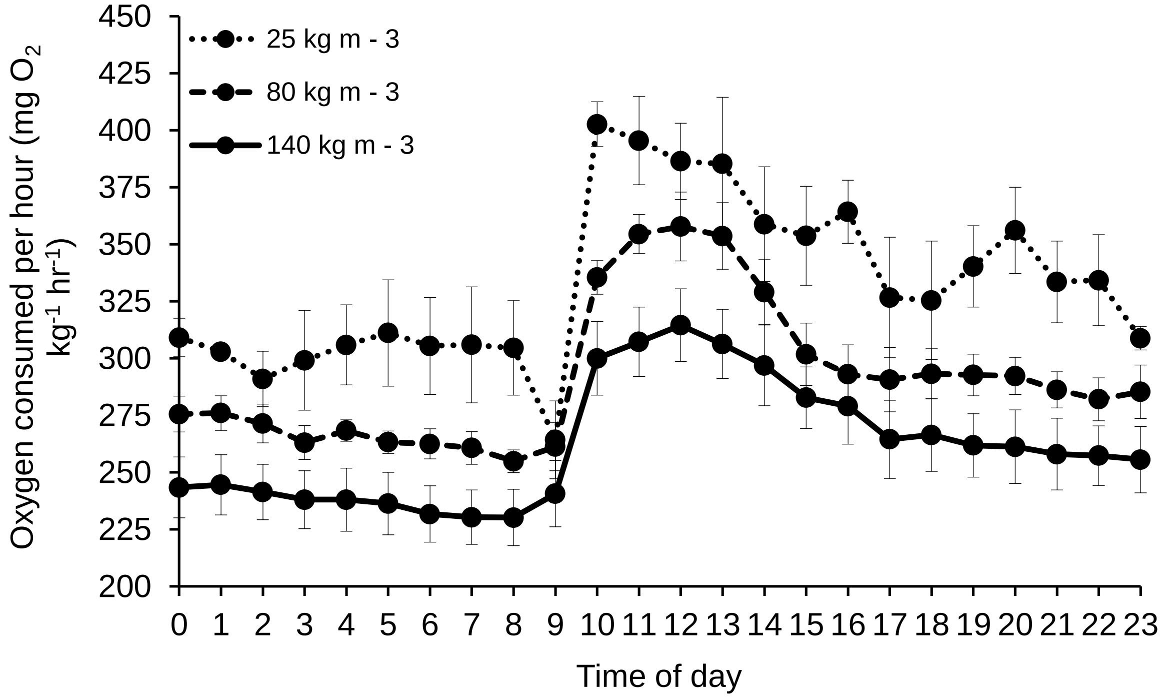

Figure 9. The hourly oxygen consumption rates (mg O2 kg^{-1} hr^{-1}) between the three density treatments (n=3 tanks per treatment); 25 kg m^{-3}, 80 kg m^{-3} and 140 kg m^{-3}, normalised for a 205 gram fish. The days during which the fish grew from 190 grams to 220 grams are used.

The total scales lost per kilogram of fish (g kg^{-1}) during the period when the fish in each tank experienced similar growth rates was used to quantify the amount of scales lost. Similarly to the oxygen consumption measurements, a negative relationship between scale loss and stocking density was observed, with a decrease in total scale loss with increasing density (Fig. 10).

There is little information about the causes of scale loss. It has been speculated that scale loss has been attributed to abrasion with the environment (Abbott and Dill, 1985). However, it has been observed to be the result of aggressive interactions between conspecifics (Abbott and Dill, 1985; Neat et al., 1998). As the fish held at the density of 140 kg m^{-3} were in a crowded situation, it could be expected that they would lose a large quantity of scales if abrasion with the environment was the cause here. Instead, it is more probable that the scales lost by individuals was due to aggressive interactions, and as scale loss was low at 140 kg m^{-3}, aggressive behaviour was concluded to be reduced at this density. Additionally, the positive association between total scale loss and total oxygen consumption further strengthened the conclusion that aggressive behaviour was reduced at the density of 140 kg m^{-3}.

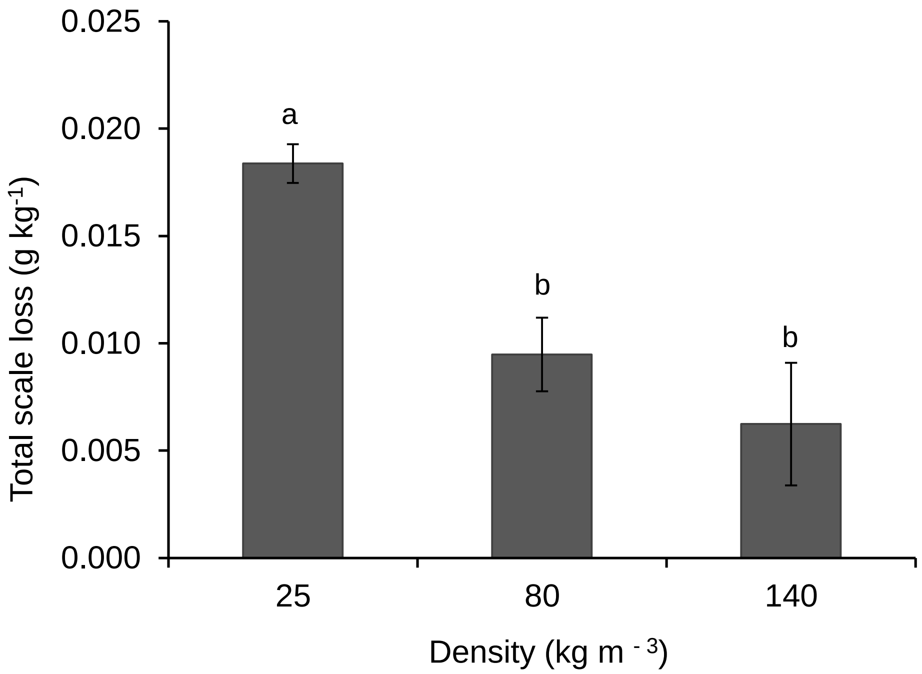

Figure 10. The total scale loss (g kg^{-1}) between the density treatments (n=3 tanks per treatment); 25 kg m^{-3}, 80 kg m^{-3} and 140 kg m^{-3} during the period (selected days) when the fish grew from from 190 grams to 220 grams. The letters (a & b) denote where the significances lie.

A degree of the fin erosion observed in certain fin types in rainbow trout has been attributed to aggressive behaviour (Abbott and Dill, 1985). However, the results for fin erosion were somewhat inconclusive on this. Of the different fin types, an effect of the density of 80 kg m^{-3} was seen on the caudal fin and an effect of the density of 140 kg m^{-3} was seen on the left pelvic fin. This does not support the theory that aggressive behaviour was reduced at the density of 140 kg m^{-3}. However, it could be that aggressive behaviour is only a minor factor affecting initial fin erosion, and that in fact other factors that increase the severity of fin erosion. Indeed, it has been well documented that fin erosion increase with increasing density due to factors such as poor water quality, abrasion with the environment and elevated stress levels (Ellis et al., 2008; Ellis et al., 2002; North et al., 2006).

There was no difference between the three densities in the concentrations of plasma cortisol (ng ml^{-1}), cortisol levels were not elevated at the highest density of 140 kg m^{-3} suggesting that the fish did not experience chronic stress at this density. This is contrary to what was expected, as high densities are considered to be a chronic stressor. However, these findings have been found in other studies in Salmonids, where concentrations can return to basal levels despite the continued presence of chronic stress (Barton et al., 1980; Pickering, 1992; Strange and Schreck, 1978) and may represent an acclimation effect (Ellis et al., 2012). Although we did not measure the cortisol response at the beginning of the experiment, we can conclude that cortisol values returned to basal levels within two weeks after initial stocking (as this was when blood samples were taken).

On the other hand, serotonergic activity levels (5-HIAA/5-HT) in the brain stem were found to increase with density and were most elevated at the highest density of 140 kg m^{-3} (Figure 10), indicating elevated stress levels in the individuals held at this density. Previous studies have shown elevated serotonergic activity levels in individuals exposed to prolonged periods of social stress (Winberg et al., 1991; Winberg et al., 1992; Winberg and Nilsson, 1993; Øverli et al., 1999). Serotonergic activity is suggested to play a stimulatory role on the HPI-axis (Winberg and Nilsson, 1993; Winberg and Lepage, 1998) and activity levels are often correlated to plasma cortisol concentrations (Øverli et al., 1999). However, during prolonged stress, HPI axis reactivity may decrease while 5-HT activity remains high (Winberg and Lepage, 1998). Therefore, low levels of plasma cortisol and elevated levels of serotonergic activity (5-HIAA/5-HT) levels indicated chronic stress at the highest density of 140 kg m^{-3}.

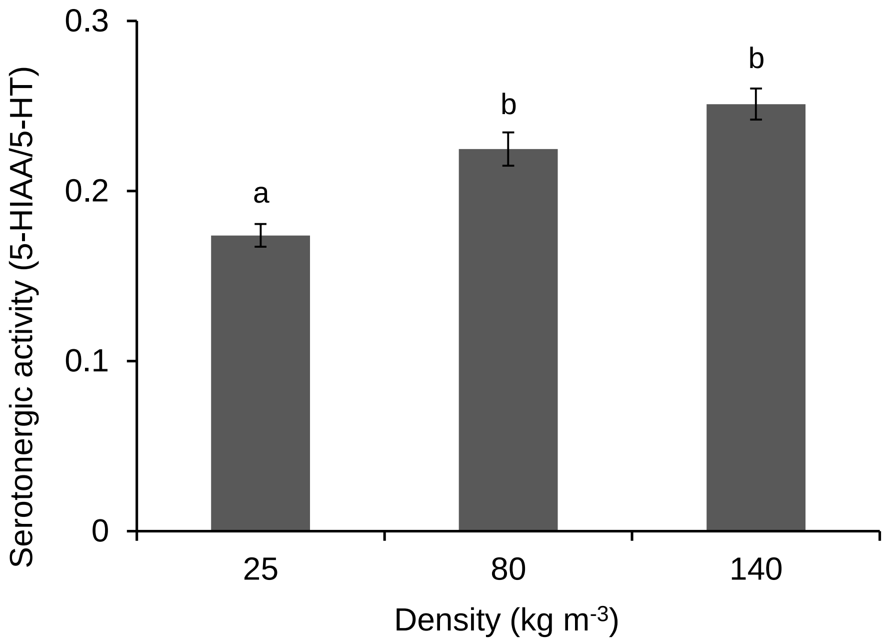

Figure 11. The serotonergic activity level (5-HIAA/5-HT) in the a) Brain stem, b) Telencephalon, and c) Hypothalamus of individuals (n=8 per tank in triplicate) between the density treatments; 25 kg m^{-3}, 80 kg m^{-3} and 140 kg m^{-3}. The letters (a & b) denote where the significances lie.

The results of the plasma cortisol concentrations (ng ml^{-1}) following recovering from an acute stressor at each of the three densities are summarised in Figure 12. As these results are unpublished, they are presented with details of statistical analysis. Basal cortisol levels before the stressor were low at all densities, falling below 5 ng ml^{-1}. Following the acute stressor (time 0), there was a striking elevation in cortisol concentration at all densities. Thereafter, there was a sharp recovery in cortisol levels with time, with values returning to pre-stressor levels within 4 hours. There was a general trend towards lower cortisol concentrations at the high density of 140 kg m^{-3} at 1 hour (time 1). At time point 1, the one-way ANOVA revealed a difference between densities (p=0.049). Specifically, the Tukey's post hoc test indicated a difference in concentrations between 25 kg m^{-3} and 140 kg m^{-3} (p=0.040), but not between 25 kg m^{-3} and 80 kg m^{-3}, or 80 kg m–3 and 140 kg m^{-3}. This suggests that at the density of 140 kg m^{-3} there was a faster down regulation of the HPI axis following the acute stressor, resulting in less cortisol release into the bloodstream. Chronic stress can result in down regulation of the HPI axis through negative feedback mechanisms (reviewed by Wendelaar Bonga, 1997). Furthermore, as the HPI axis has been found to be influence by neurotransmitters such as 5-HT (Winberg and Nilsson, 1993; Winberg et al., 1997) previous chronic exposure to stress by the fish as 140 kg m^{-3} may have influenced the faster down regulation of the HPI axis following the acute stressor. As such, neurochemical measures such as 5-HT-ergic activity and the expression of genes involved in HPI axis down regulation, such as 5-HT receptors, may reveal central mechanisms which are involved in differences in ability to down regulate the stress induced cortisol response.

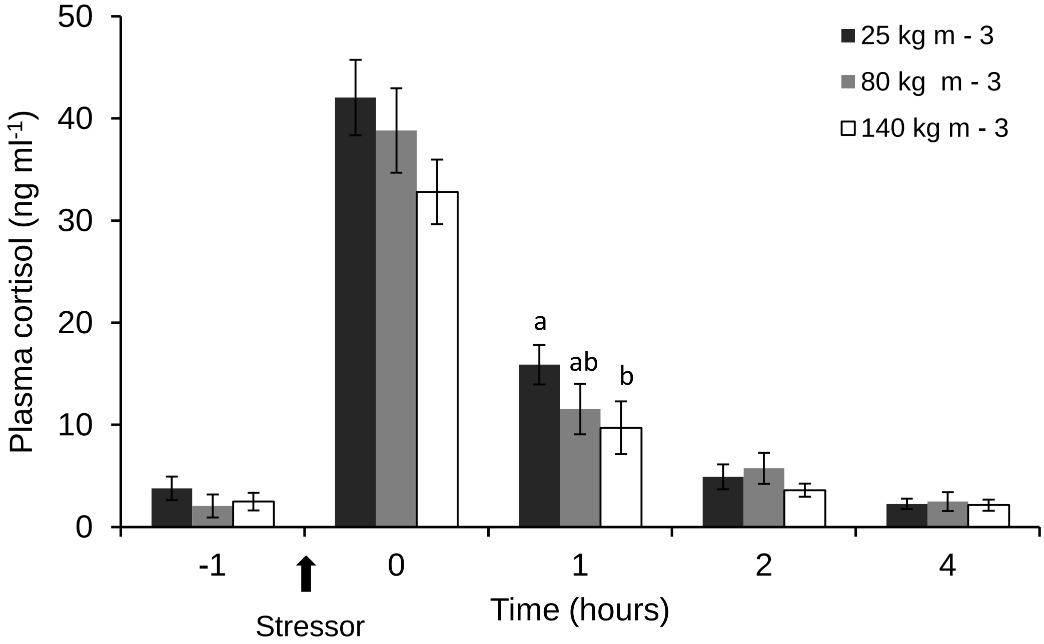

Figure 12. Plasma cortisol concentrations of individuals held at three different densities (25, 80 and 140 kg m^{-3}), during recovery from an acute stressor. N=12 individuals per density. (Concentrations analysed using Radioimmunoassay by Maria Moltesen, PhD student, DTU Aqua)

The total energy dissipated for metabolism (Kj kg^{-1}) was used as an indicator of energy expenditure. The lower total energy dissipated for metabolism at the density of 140 kg m^{-3} suggested that energy expenditure was reduced at this density (Figure 13). Aggressive social interactions between conspecifics in Salmonid species have been suggested to be energetically costly (Lefrancois et al., 2001; Li and Brocksen, 1977). This goes together with the theory that aggressive behaviour was reduced at the density of 140 kg m^{-3}, as indicated by the reduced oxygen consumption rates and scale loss observed at this density. The implications of the reduced energy expenditure at 140 kg m^{-3} was a tendency for a more efficient utilisation of feed and hence improved growth performance at this density (Figure 13).

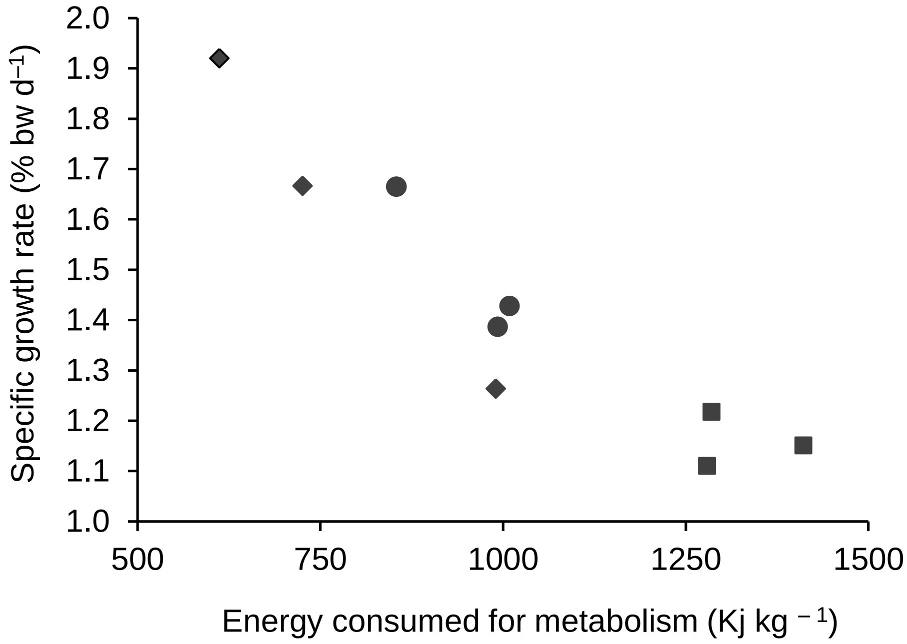

Figure 13. The relationship between the specific growth rate (% bw d^{-1}) and the total energy consumed for metabolism (Kj kg^{-1}) during the selected period when the fish grew from 190 to 220 grams. Triangles denote the 25 kg m^{-3} tanks, circles the 80 kg m^{-3} tanks, and squares the 140 kg m^{-3} tanks (n=3 tanks per density).

Chapter 3: Discussion of findings

6. Overall conclusions

In this section I will finish with a summary the main conclusions, limitations and future perspectives of this work.

To sum up, the overall objective of the PhD project was to investigate aspects of welfare related to rearing density in farmed rainbow trout, using behavioural and physiological methods. Specifically, there are two main aspects that have been presented and discussed in the current work. This has been:

1) The establishment and application of a novel behavioural method using the spatial distribution of fish in two-tank systems stocked at different densities to establish an optimal density limit, and;

2) Investigating indicators of welfare, using mainly a function-based physiological approach, to make conclusions about the welfare status of the fish at the density limit established using the behavioural method.

The main findings of each aspect are highlighted below:

6.1. Highlights

1) Behavioural method:

- There was a lower percentage of fish occupying the "crowded" tank of the two-tank system stocked at the highest density of 80 kg $m^{-3;}$
- Neuroendocrine indicators of stress reflected crowding stress or aversion in the individuals in this tank of the two-tank system;
- This level of aversiveness to crowding was shown at an absolute density of approximately 140 kg m^{-3}.

2) Physiological methods:

- At this density of 140 kg m^{-3}, the lower oxygen consumption rates suggested reduced levels of social hierarchy related aggressive encounters;

- The lower quantity of scale loss collected from the tanks stocked at this density further supported the suggestion that aggressive behaviour was reduced at 140 kg m^{-3};
- Higher brain serotonergic activity in the brain stem of individuals held at this density indicated elevated stress levels, despite low concentrations of plasma cortisol;
- A lower energy expenditure at 140 kg m^{-3} was attributed to reduced aggressive social interactions between individuals;
- This was linked to a tendency for better utilisation of ingested feed and hence growth performance at 140 kg m^{-3}.

6.2. Implications

Based on the main findings highlights above, it could be asked what can be concluded about the welfare status of the fish held at the density that was revealed using the behavioural method?

The findings revealed three main welfare aspects of being held at the density limit of 140 kg m^{-3}. The first was the presence of chronic stress, the second was reduced levels of aggressive social interactions, and the third was the link between energetic expenditure and growth performance.

Stress levels were elevated at this density of 140 kg m^{-3}, as indicated by elevated serotonergic activity in the brain stem, although plasma cortisol values were low. This may be indicative of a chronic crowding situation. However, evidence for the association between high density or crowded conditions and chronic stress has been difficult to establish in previous research. Reviewing all the studies to date, a range of effects of density on indicators of the primary stress response, mainly cortisol, have been found. For example, a study by Pickering et al. (1991) found an effect of high density on plasma cortisol concentrations. Likewise, Pickering & Pottinger (1987) and Leatherland (1993) found an effect of high density on plasma cortisol levels. However, the former study found that the concentrations were only elevated for the first 10 days (Pickering and Pottinger, 1987) and the latter that concentrations were only elevated in a few samples (Leatherland, 1993). On the contrary, Leatherland and Cho (1985) and North et al. (2006) showed that plasma cortisol levels were higher at low density, attributed in the latter study to social stress during the establishment of territories and dominance hierarchies (North et al., 2006). Furthermore, a few studies have found no effect. In a study by Person-Le Ruyet, no effect of density on physiological measures was found. Nevertheless, the general consensus is that crowding is a chronic stressor in rainbow trout (Ellis et al., 2002), despite the lack of clear evidence from indicators of the primary stress response, and that the resulting chronic or elevated stress levels are detrimental to welfare.

At this density of 140 kg m^{-3}, it was concluded that social hierarchy related aggressive interactions, as indicated by reduced oxygen consumption and scale loss, were reduced. This is in line with previous evidence concerning the relationship between aggressive behaviour and density. There is evidence that by increasing density, it is possible to alter aggressive territorial behaviour to schooling type behaviour in Salmonids (Grand and Dill, 1999). This is due to the fact that establishing and maintaining ordered dominance hierarchies at high density becomes difficult, thereby decreasing the intensity of aggressive acts (Alänärä and Brännäs, 1996; Bagley et al., 1994; Li and Brocksen, 1977). Indeed, it could be speculated that due to the confined conditions at this density, the fish were unable to display the natural (aggressive) behaviour and were forced to adopt shoaling behaviour. As a result, the negative impacts of aggressive behaviour on welfare were reduced.

Furthermore, the reduced aggressive behavioural interactions were suggested to be associated with the lower levels of energy expenditure at the density of 140 kg m^{-3}. In addition, this was linked to a tendency for better utilisation of ingested feed and hence growth performance at this density. This suggests that growth performance, as an indirect indicator of the maladaptive consequences to crowding, did not show reduced welfare at this density. This is in contrast to the general consensus formed by the majority of the other studies investigating the effect of density on growth performance in rainbow trout. For example, Boujard et al. (2002) concluded that growth rate was decreased at a high density of 100 kg m^{-3}. Furthermore, in a study by Person-Le Ruyet et al. (2008), growth performance was worst at the highest density of 120 kg m^{-3}. On the other hand, no effect of density on growth performance was found at a density of 80 kg m^{-3} (North et al., 2006).

Taken together, despite the chronic stress levels at this density, it could be speculated that due to reduced aggressive behaviour there was a bioenergetic advantage of crowding at this density level, revealed by the fish in the two-tank system, of 140 kg m^{-3}. Furthermore, it may be concluded that application of the method using the two-tank systems therefore provided new insight into an optimal stocking density limit for rainbow trout. Potential for further development of the method is described in section 6.4.

6.3. Limitations

Upon reflection, there are several aspects of the current work that could be improved upon, given the opportunity.

Firstly, if experiment 1 were to be repeated, we would quantify the aggressive behaviour displayed by the dominant aggressive individuals in the “dominant” tank to confirm their social status. This would also give us information about what was actually happening in this tank, such as; whether the same fish always occupy the tank, if the levels of aggression were always constant, what the pattern of aggression was towards the fish entering the tank.

Furthermore, as water quality is one of the factors influencing the effects of density on welfare, it may have affected the spatial distribution of the fish in the two-tank systems. Although water quality parameters were checked at a system level daily, they were not measured specifically at the tank level. It may be speculated that as there was such a high number of fish in the “crowded” tank of the two-tank system stocked at the highest density, the water quality may have been influenced. Therefore, the density effects observed on the neuroendocrine stress levels could be, in part, influenced by water quality. Therefore, it would be beneficial to do additional studies to exclude the influence of this factor.

For experiment 2, additional indicators of stress could have been used, such as from the secondary stress response such as, for example, plasma glucose and haematocrit. This may have given a more complete picture of the reactivity of the stress response at the different densities. Furthermore, despite its’ popularity as an indicator of stress, plasma cortisol can be an unpredictable indicator of chronic stress, especially in relation to stocking density, and contrasting results have been documented in the literature.

Additionally, it would have been relevant to have used a fourth density in experiment 2 of 40 kg m^{-3} reflecting the total absolute density in the “crowded” tank of the two-tank system stocked at 20 kg m^{-3}. As the reasoning behind using the density of 80 kg m^{-3} as the intermediate density was that it reflected the total absolute density in the “crowded” tank of the two-tank system stocked at 40 kg m^{-3}, it would have been interesting to do also this for the system stocked at the lowest density (20 kg m^{-3}).

6.4. Future perspectives

The method presented in this PhD project provides a promising approach for using the spatial distribution of fish in two-tank systems to determine density levels in rainbow trout.

As mentioned, a subsequent goal would be to further develop the current method. This could be done by refining the present experimental methodology (as described in section 6.3.). Furthermore, the method could be elaborated on to generate results that would be applicable to a range of culture situations. Indeed, a single maximum density limit for rainbow trout, as presented in this PhD work, would not be appropriate for application to all culture systems. This is due to the fact that the effects of density are specific to the environmental conditions provided during the study (Ellis et al., 2002).

To illustrate this, in our study using spatial distribution in two-tank systems, a result that was found was that there was a positive relationship between density and fish weight. This suggested that larger fish accepted to be at a higher density than smaller individuals. Additionally, there was a negative relationship between fish weight and serotonergic activity in the brain stem. This suggested that of the fish that accepted to stay in the “crowded” tank, the smaller fish had higher stress levels compared to larger fish. Taken together, this highlighted the fact that fish size had an influence on the distribution pattern of the fish in the two-tank systems and that this was an important factor to consider when investigating critical densities. Hence, it would be applicable to do additional studies to determine how fish size influences the distribution of fish in the two-tank systems and. Furthermore, from a practical point of view, it would be beneficial to establish critical density limits for rainbow trout under diverse culture conditions; for example, fish of different sizes and ages, given different feeding levels and held at different temperatures.

On this line, it would also be interesting to look at within species differences, in terms of life history and at a population level with individual differences (i.e. personality or coping style) and how this would influence the spatial distribution of the fish in the two-tank system and ultimately the level of crowding experienced as aversive. The High- (HR) and Low- (LR) responsive trout model, established by Dr Tom Pottinger, may provide such an opportunity, as they resemble two extremes in behavioural and physiological profile. One could use populations of these strains and identify how they differ in their spatial distribution in two-tank systems and level of aversion to crowding.

Ultimately, the method could be extended to other relevant species. Finally, one could also speculate about whether this type of experimental set up could be used to study other welfare related issues. The two-tank system provides the potential for the fish to indicate environmental conditions that may be favourable.

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Appendix I

Paper I: Utilising spatial distribution in two-tank systems to investigate a threshold for crowding in rainbow trout *Oncorhynchus mykiss*

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Utilising spatial distribution in two-tank systems to investigate the level of aversiveness to crowding in farmed rainbow trout *Oncorhynchus mykiss*

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ABSTRACT

In aquaculture, fish are exposed to a range of unfavourable environmental conditions. Amongst these, stocking density has attracted considerable attention as inappropriate densities may compromise welfare and negatively impact production. However, the recommendations for stocking remain elusive. The aim of the present study was to apply a novel method to investigate a level of crowding that indicated aversiveness in rainbow trout (*Oncorhynchus mykiss*). In a two-tank system, where two identical tanks were connected via a doorway, it was observed that social behaviour controlled the distribution of the fish between the tanks. Fish were stocked at equal quantities in each tank of the system. The doorway was opened and the fish moved between the two tanks. Typically, this resulted in one tank being occupied by a few highly aggressive dominant individuals ("dominant" tank) and the majority of the fish occupying the second tank ("crowded" tank). Here, the potential of this unequal spatial distribution for quantifying aversion to crowding was explored. Fish were stocked in three two-tank systems at a total density of 20, 40 and 80 kg m^{-3} respectively. The number of fish in each tank was determined every three days throughout the duration of the experiment and the percentage of fish in the "crowded" tank was used as an indicator of the distribution pattern in the two-tank systems. The results indicated a negative relationship between the total density stocked (20, 40 and 80 kg m^{-3}) and the percentage of fish in the "crowded" tank. A subsample of individuals was sacrificed for blood and brain samples every three days from the "crowded" tank, prior to the fish count. The neuroendocrine indicators of stress, elevated serotonergic activity levels which were not associated with high plasma levels of cortisol, suggested chronic stress in the fish at the highest total density stocked (80 kg m^{-3}). Taken together, these results indicated that a level of aversiveness to crowding had been reached at the highest density stocked, where the mean absolute density, irrespective of time of day, observed in the "crowded" tank was 126.5 ± 3.7 kg m^{-3}.

1. Introduction

Stocking density in aquaculture has received considerable attention in recent years. This has been the consequence of an increasing public concern for the

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welfare of fish from aquaculture (Huntingford et al., 2006) and recognition in the commercial and scientific communities that inappropriate densities contribute to a reduced welfare status in fish (Ellis et al., 2002).

The general perception is that welfare decreases with increasing density, though there are no unanimous results of the effect of increasing stocking densities on indicators of welfare, such as general performance and stress hormone levels (Ellis et al., 2002). Naturally, this may in part be due to species differences, where welfare may be optimal for some species at higher densities and for others at lower densities. However, contradictory results have been found even within a species (Ellis et al., 2002; Brännäs and Johnsson, 2008). This has been attributed to differences between studies in experimental design and methodology (Ellis et al., 2002). However, it has also highlighted the fact that stocking density is a complex issue and the negative effects on welfare are likely to be the cause of a combination of factors as a consequence of stocking density (Bagley et al., 1994; Person-Le Ruyet et al., 2008), such as water quality and social interactions (Ellis et al., 2002).

The method that has most commonly been used to study the relationship between stocking density and welfare has been by investigating the effects of varying density levels on indicators of welfare; such as performance, condition, health and stress levels (Boujard et al., 2002; Ellis et al., 2002; Larsen et al., 2012; McKenzie et al., 2012; North et al., 2006; Person-Le Ruyet et al., 2008; Skøtt Rasmussen et al., 2007). Through such studies it has been possible to make general conclusions about the influence of stocking density on welfare. Ellis et al. (2002) reviewed all the studies to date that had investigated the relationship between stocking density and welfare for rainbow trout, *Oncorhynchus mykiss*. They concluded that despite the lack of clear evidence, high stocking density had the potential to reduce welfare. Since then, additional studies have been carried out, which concluded that low as well as high stocking densities had the potential to compromise indicators of welfare (Boujard et al., 2002; Ellis et al., 2002; Larsen et al., 2012; McKenzie et al., 2012; North et al., 2006; Person-Le Ruyet et al., 2008; Skøtt Rasmussen et al., 2007).

A number of the published studies on this issue have attempted to make specific recommendations for maximum stocking densities for rainbow trout based on their experimental results (Ellis et al., 2002). Depending on the type of rearing system, the recommendations for appropriate stocking densities made by the studies reviewed ranged from 4 to more than 267 kg m^{-3} (Ellis et al., 2002). Evidently, concrete conclusions regarding the density limits at which welfare and production in rainbow trout are optimised continue to be ambiguous. Therefore, developing alternative methods to investigate the density levels that fish experience as critically crowded may provide insight into optimal density limits for rainbow trout.

The aim of the current study was to apply a novel method to investigate a level of aversiveness to crowding of farmed rainbow trout (*O. mykiss*). This was achieved by studying the spatial distribution in two-tank systems stocked fish at different densities to establish a level of aversion to crowding. Here, a two-tank system consisted of two identical tanks which were attached to each other with a doorway, allowing individuals to move freely between the two tanks. Groups of fish held in this system were observed to distribute themselves unequally between the two tanks, despite equal initial stocking and equal feed rations in the two tanks. Social behaviour was established as the controlling factor for this distribution pattern, as aggression and dominance related behaviours by a few individuals in one tank, referred to as the "dominant" tank, drove the majority of the group into the second tank, referred to as the "crowded" tank. The percentage of fish, of the total quantity of fish in the system, occupying the "crowded" tank was used as an indicator of the distribution pattern between the two tanks at three stocking densities; 20, 40 and 80 kg m^{-3}. To support these observations, neuroendocrine indicators of stress, plasma cortisol and brain serotonergic activity, of individuals from the "crowded" tank were examined to determine crowding stress.

2. Materials and methods

2.1. Experimental fish

Rainbow trout from Mark Mølle fish farm, Nykøbing Mors in Denmark were used in the present study. The fish were transported by truck to the Danish Technical University, Institute of Aquatic Resources (DTU Aqua) in Hirtshals and upon arrival unloaded directly into quarantine tanks. While in quarantine, the fish were put on a feeding regime at 0.75% of their total body mass per day. Additionally, the salt content in the water was slowly increased to 15‰. The fish were held in quarantine conditions for a period of 15 days, after which they were available to be used for experiments.

Fish were ordered and delivered on two occasions to provide adequate quantities of individuals for all three trials of the experiment. Fish from the first delivery were used in trial 1 and 2 and fish from the second delivery were used in trial 3. The fish originated from the same family.

At the time of arrival, the fish from the first delivery had an average individual weight of 150 g. At the time the fish were used during trial 1 and trial 2, the fish had an average individual weight of 279 g and 390 g, respectively. At the time of arrival, the fish from the second delivery had an average individual weight of 300 g. At the time the fish were used during trial 3, the fish had an average individual weight of 430 g.

2.2. Experimental facilities

The three trials of the experiment were carried out using two-tank systems. Each system consisted of two identical 700 l circular tanks attached to one another via a doorway. The doorway could be opened by the researcher by removing the sliding door. Each tank was 100 cm in height and had a diameter of 100 cm. The doorway had a width of 15 cm and ran the height of the tank. Each tank was individually equipped with a water inflow and outflow, as well as an oxygen and air supply. A water current of approximately 0.5 BL s^{-1} (body lengths per second) was achieved through small holes in inflow pipe creating pressure, thereby circulating the water around the tank.

Three two-tank systems, standing parallel to each other, were used simultaneously during each trial and were supplied with water from the same recirculating system. The water quality parameters in the system; temperature, ammonia, nitrate, nitrite and pH were checked daily to ascertain that they were within optimal levels for the fish. The temperature of the water in the system was $16 \pm 0.01\,^{\circ}C$, ammonia (NH_3/NH_4^+) levels were $0\,mg\,l^{-1}$, nitrite (NO_2^-) and nitrate (NO_3^-) were 37.6 ± 1.9 and $0.4 \pm 0.05\,mg\,l^{-1}$ respectively and pH was 7.6 ± 0.01. Oxygen levels were adjusted manually as the fish moved between the tanks and kept at levels between 90 and 100% saturation in both tanks of each system. The fish were on a 12/12 hour light dark regime, with the lights switching on automatically at 08:00 and switching off at 20:00.

2.3. Experimental design

During pilot studies it was observed that when groups of fish were placed in a two-tank system, the result was an unequal distribution of individuals between the two tanks. At the start, an equal quantity of fish was stocked in each tank of the system. Each tank was given the same amount of feed, throughout the study. The doorway was opened allowing individuals to move freely between the two tanks. The resulting distribution pattern typically observed was one tank becoming occupied by a few dominant aggressive individuals and the majority of the fish occupying the second tank. The few dominant individuals occupying one tank drove out the majority of the group into the second tank, thereby controlling the distribution of the group in the two-tank system. Although quantifications of their behaviour were not made, observation of the fish confirmed that they exhibited behaviour that was characteristic for a dominant individual. They displayed territorial behaviour, monopolising the food resource with chasing out individuals entering the tank. Furthermore, if more than one individual was present they displayed agonistic behaviour towards each other. The tank occupied by the dominant individuals will be referred to as the “dominant” tank and the tank holding the majority of the fish as the “crowded” tank. For the present study, we utilised this inequality in the distribution pattern of groups of fish in the two-tank system to investigate a level of aversiveness to crowding.

Three stocking densities were used during the experiment; the first two-tank system was stocked at $20\,kg\,m^{-3}$, the second at $40\,kg\,m^{-3}$ and the third at $80\,kg\,m^{-3}$. The experiment was completed in triplicates as trial 1, trial 2 and trial 3. Between each trial, the stocking density in each two-tank system was changed. The number of fish in each tank was determined every three days during the experiment for a period of two weeks. As the distribution of the fish changed between the night time and the day time, the fish count and sampling of individuals in the “crowded” tank was determined at two time points during the daily cycle. One time point was chosen at the end of the night time hours, which was in the morning at 07:30 when it was still dark. The second time point was chosen at the end of the day time hours, which was in the evening at 19:30 when it was still light. These time points will be referred to as in “dark” and “light”, respectively. During the experiment, sampling was alternated between the morning (“dark”) and the evening (“light”). Between each trial, the order of the sampling time points was changed. If in trial 1, the first sampling was done in the “dark”, then during trial 2, the first sampling was done in the “light” and so on. For each trial, there were a total of four sampling sessions; two at “dark” (session 1 and session 2) and two at “light” (session 1 and session 2).

Additionally, a subsample consisting of six individuals from the “crowded” tank was sampled for blood and brain parts. The individuals were taken before the number of fish in the tank was determined. Plasma cortisol concentrations and brain serotonergic activity were analysed to assess the stress levels in this tank. Cortisol is a commonly used physiological indicator of stress in fish when studying the effects of stocking density (Ellis et al., 2002; North et al., 2006). Additionally, serotonergic activity, the ratio between the brain tissue concentration of serotonin (5-HT, monoamine) and 5-hydroxyindoleacetic acid (5-HIAA, metabolite), has previously been used as an indicator of stress in relation to stocking density in rainbow trout (McKenzie et al., 2012) and has also been used as an indicator of chronic social stress in salmonid fish in pairs and small groups (Øverli et al., 1999; Winberg et al., 1991, 1992; Winberg and Nilsson, 1993).

2.4. Experimental procedure

The fish were transported to the experimental facility and stocked into the three two-tank systems using 20, 40 and $80\,kg\,m^{-3}$. The two tanks of each system were stocked with equal densities. During this initial stocking process, the number of fish going into each tank was counted to allow for future determination of the percentage of fish occupying each tank. After initial stocking, the fish were given an acclimation period of a week and the doorway separating the two tanks was left closed to hinder any redistribution before the start of the experiment. The fish in each tank of the systems were fed at 1% of their total body weight (grams) from 08:00 to 20:00 using 12 h automated belt feeders. After an acclimation period of a week, the doorway between the two tanks in each system was opened, allowing the fish to swim freely between the two environments. The amount of feed given to each tank of the two-tank systems was kept at the same level as during the acclimation period.

The number of fish in each tank was determined every three days. For practical reasons, this was done by counting the number of fish in the “dominant” tank and subtracting this count from the total number of known fish in the system. Before determining the number of fish in each tank, a subsample of six individuals from the “crowded” tank of each system were sacrificed by an overdose of anaesthetic (Ethylene glycol monophenyl ether). Blood samples were collected from the caudal vein using 1 ml syringes filled with EDTA (ethylenedinitrilotetraacetic acid disodium salt dihydrate) powder. The blood samples were centrifuged and the plasma was separated into 1 ml eppendorf tubes and frozen at −80 °C for later analysis. Whole brains were dissected out from each fish and separated into four parts; brain stem, hypothalamus, telencephalon and optic lobes, frozen directly using liquid nitrogen and then stored in the −80 °C freezer for later analysis.

2.5. Analysis of plasma cortisol and serotonin

Cortisol was extracted from the plasma using ethyl ether, evaporated using a vacuum centrifuge and re-suspended in an extraction buffer (ELISA kit extraction buffer). Concentrations (ng ml^{-1}) were quantified using the ELISA kit standard method (Neogen, Product No. 402710).

Frozen brain parts were homogenised in a homogenising reagent (4% perchloric acid, 0.2% ethylenediaminetetraacetic acid, 40 ng ml^{-1} dihydroxi benzylamine hydroxide solution) and centrifuged at 10,000 rpm at 4 °C for 10 min to separate the supernatant. The supernatant was assayed using high performance liquid chromatography (HPLC) with electrochemical detection, described in Andersson and Höglund (2012), to quantify 5-HIAA (metabolite) and 5-HT (monoamine). The supernatant (sample) was transported through the HPLC system by a mobile phase, which consisted of a buffer solution containing 10.35 g l^{-1} sodium phosphate, 0.3252 g l^{-1} sodium octyl sulphate, 0.0037 g l^{-1} ethylenediaminetetraacetic acid disodium salt dehydrate, 7% acetonitril in deionised water. The compounds in the sample were analysed using a computer program (software; Clarity, DataApex Ltd.). The sample 5-HIAA and 5-HT quantities were compared with quantities from solutions of known concentration (standards) to determine the actual concentrations.

2.6. Statistical analyses

The percentage of the total number of fish in one tank was used as a measure of crowding. The difference in the proportions of fish occupying the "crowded" tank between density treatments (20, 40 and 80 kg m^{-3}), sampling time ("dark" and "light"), trial (1, 2 and 3), two-tank system (1, 2 and 3) and session (1 and 2) was analysed with a generalised linear model (GENMOD). In addition to the mentioned variables (class variables) initial weight of the fish was used as a covariate. The response variable was number of fish in the crowded tank/total number of fish (binomial distribution).

To determine if there was a difference in the concentrations of plasma cortisol, concentrations of 5-HIAA and 5-HT, and ratios of 5-HIAA/5-HT between density treatments (20, 40 and 80 kg m^{-3}), sampling time ("dark" and "light"), trial (1, 2 and 3), and session (1 and 2), was determined using an ANCOVA, with fish weight (at the time of sampling) as the covariate. The log concentrations of plasma cortisol, log concentrations of 5-HIAA and 5-HT, or arcsin ratios of 5-HIAA/5-HT were used as the dependent variables. A Tukey's post hoc test was used to determine between which treatments the significances occurred.

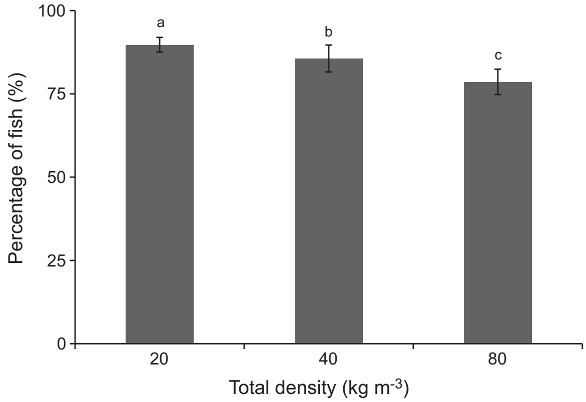

Fig. 1. The percentage of fish in the "crowded" tank of the two-tank system between the three total densities ($n = 3$). The letters (a, b and c) indicate a significant difference between treatments.

3. Results

3.1. Spatial distribution of fish

3.1.1. Percentage of fish in the "crowded" tank

The GENMOD did not indicate any differences between trials ($p = 0.986$), two-tank system ($p = 0.343$), sampling time ($p = 0.143$) or session ($p = 0.875$). The percentage of the fish choosing to be in the crowded environment decreased with increasing total stocking densities ($p < 0.001$, Fig. 1), with a significant difference between stocking densities 20 and 40 kg m^{-3} ($p = 0.007$), between 20 and 80 kg m^{-3} ($p < 0.001$) and between 40 and 80 kg m^{-3} ($p < 0.001$). At 80 kg m^{-3}, of a total of 314 ± 23 individuals in the system, 251 ± 27 occupied the "crowded" tank. At 40 kg m^{-3}, 125 ± 11 out of a total of 144 ± 9 individuals occupied the "crowded" tank. At 20 kg m^{-3}, 64 ± 6 out of a total of 77 ± 7 individuals occupied the crowded tank. Furthermore, there was a positive relationship between initial fish weight and density in the crowded tank ($p < 0.001$).

3.1.2. Absolute density in the "crowded" tank

The absolute density (kg m^{-3}) in the "crowded" tank of the two-tank systems was determined from the percentage of the fish occupying this tank. At stocking density 20 kg m^{-3} the mean absolute density in the "crowded" tank irrespective of sampling time was 32.5 ± 1.5 kg m^{-3} (Fig. 2). At "dark" and "light" the absolute density was 30.7 ± 2.3 kg $m^{-3}$and 34.3 ± 2.1 kg m^{-3}, respectively. At 40 kg m^{-3} the mean absolute density was 63.7 ± 2.4 kg m^{-3} (Fig. 2), and 57.4 ± 3.5 kg m^{-3} and 69.9 ± 3.3 kg m^{-3} in the "dark" and "light", respectively. At 80 kg m^{-3} the mean absolute density was 126.5 ± 3.7 kg m^{-3} (Fig. 2), and in the "dark" and "light" was 115.7 ± 5.5 kg m^{-3} and 137.4 ± 10.0 kg m^{-3}, respectively.

3.2. Neuroendocrine indicators of stress

3.2.1. Plasma cortisol

Despite a tendency for slight elevation in the plasma cortisol concentrations of individuals in the "crowded" tank at the highest total density stocked (kg m^{-3}), there

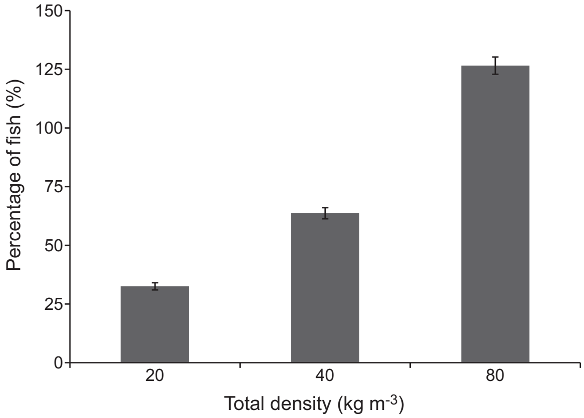

Fig. 2. The absolute density (kg m^{-3}) in the “crowded” tank at each total density ($n = 3$).

was no difference in the levels between the three densities stocked (20, 40 and 80 kg m^{-3}; $p = 0.314$; Fig. 3). There was also no significant difference between the “dark” and “light” (sampling time; $p = 0.140$), between the first and second sampling session (session; $p = 0.077$), between trials ($p = 0.948$), two-tank system ($p = 0.128$) or fish weight ($p = 0.217$).

3.2.2. Brain ratios (5-HIAA/5-HT)

Generally, the serotonergic activity in the brain stem of the individuals in the “crowded” tank was higher in the “light” compared to the “dark” irrespective of stocking density ($p = 0.013$) and higher in the first sampling session compared to the second sampling session irrespective of density (session; $p = 0.001$). Moreover, there was a higher activity level in the individuals in the “crowded” tank of the system stocked at 80 kg m^{-3}, compared to the individuals in the two systems stocked at 20 and 40 kg m^{-3} ($p < 0.001$; Fig. 4A). Specifically, there were no differences in activity levels between 20 and 40 kg m^{-3} ($p = 0.953$), but differences between 20 and 80 kg m^{-3} ($p < 0.001$) and between 40 and 80 kg m^{-3} ($p < 0.001$; Fig. 4A). Furthermore, there was an effect of trial ($p = 0.028$). Fish weight showed a negative relationship with serotonergic activity ($p = 0.004$).

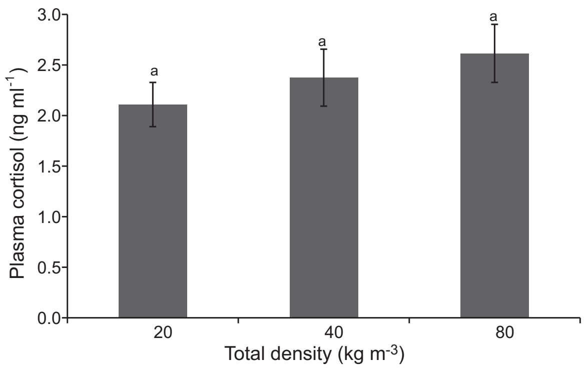

Fig. 3. Plasma cortisol concentrations of individuals taken from the “crowded” tank at each total density ($n = 18$).

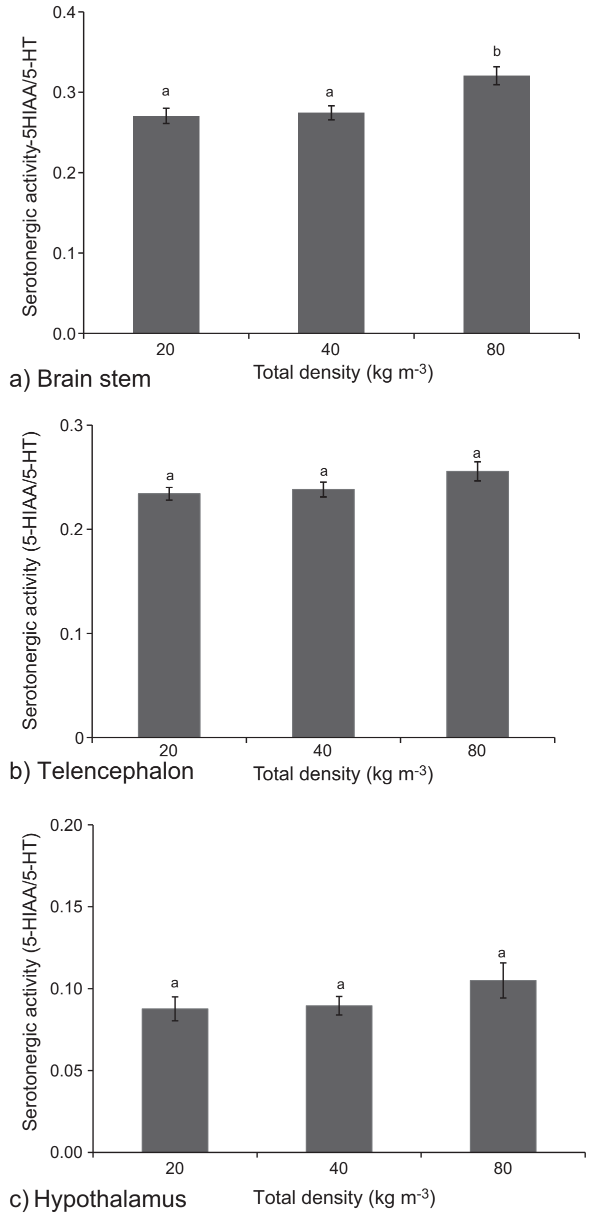

Fig. 4. Serotonergic activity (5-HIAA/5-HT) in the (A) Brain stem, (B) Telencephalon and (C) Hypothalamus of individuals ($n = 18$) in the “crowded” tank at each total density. The letters (a and b) indicate a significant difference between treatments.

The serotonergic activity in the telencephalon of the individuals in the “crowded” tank followed a similar pattern. Activity levels were higher in the individuals in the “light” compared to the “dark” irrespective of density (sampling time; $p \leq 0.001$). In contrast to the brain stem, serotonergic activity was higher in the second sampling session compared to the first (session; $p \leq 0.001$). Furthermore, in the telencephalon there was only a trend towards higher serotonergic activity in the individuals in the “crowded” tank of the system stocked at 80 kg m^{-3}, compared to 20 and 40 kg m^{-3} ($p = 0.064$; Fig. 4B). There was no effect of trials ($p = 0.919$) or fish weight (0.518).

Table 1
The concentrations (mean ± SEM) of monoamine and metabolites in the different brain regions of the individuals (n = 18) in the "crowded" tank at each total density.

Brain region	Metabolite and metabolite	Density treatment (kg m$^{3-1}$)			
		20	40	80	p value
Brain stem	5-HIAA	363.1 ± 21.0	404.5 ± 23.4	438.9 ± 19.7	0.013
	5-HT	1419.5 ± 89.3	1574.7 ± 113.6	1444.9 ± 67.7	0.653
Telencephalon	5-HIAA	1094.9 ± 55.7	1161.1 ± 51.3	1077.8 ± 47.9	0.398
	5-HT	4954.33 ± 297.4	5201.8 ± 277.9	4550.1 ± 230.2	0.190
Hypothalamus	5-HIAA	390.5 ± 17.3	410.7 ± 25.8	457.8 ± 29.5	0.439
	5-HT	5589.9 ± 373.0	5326.3 ± 372.0	5633.1 ± 420.9	0.850

The 5-HTergic activity in the hypothalamus of the individuals in the "crowded" tank, of all systems combined, did not differ between the "dark" and "light" (sampling time; $p = 0.127$), between the first and second sampling session ($p = 0.064$), between trial ($p = 0.058$), fish weight ($p = 0.109$) or the total densities stocked ($p = 0.263$; Fig. 4C).

3.2.3. *Brain 5-HT and 5-HIAA*

The concentration of the main metabolite (5-HIAA) of serotonin and monoamine serotonin (5-HT) in the brain stem, telencephalon and hypothalamus between the three density treatments (20, 40, and 80 kg m^{-3}) are given in Table 1.

In the brain stem, there was a significant effect on 5-HIAA concentration by sampling time ($p = 0.013$), session ($p = 0.001$), density treatment ($p = 0.012$) and trial ($p = 0.011$), but there was no effect of fish weight ($p = 0.468$). There was a significant difference in 5-HT concentration between session ($p < 0.001$) and trial ($p = 0.001$), but not sampling time ($p = 0.301$), fish weight ($p = 0.368$) or density treatment (0.703).

In the telencephalon, there was a difference in 5-HIAA concentration between sampling time ($p = 0.012$), but not between trials ($p = 0.069$), session ($p = 0.975$), fish weight ($p = 0.329$) or density treatment ($p = 0.345$). A similar pattern was observed in 5-HT concentrations, where an effect of sampling time ($p < 0.001$) and trials ($p < 0.001$) was observed. However, no effect of session ($p = 0.116$), fish weight ($p = 0.846$) or density treatment ($p = 0.146$) were detected.

In the hypothalamus, there was a difference in 5-HIAA concentration between sampling time ($p = 0.008$), trials ($p < 0.001$), session ($p = 0.044$), but not fish weight (0.173) or density treatment ($p = 0.321$). In 5-HT concentrations there was a difference between session ($p \leq 0.001$), trials ($p < 0.001$), but not sampling time ($p = 0.986$), fish weight ($p = 0.643$) or density treatment ($p = 0.798$).

4. Discussion

In the present study, the distribution of the fish in the two-tank systems was unequal, irrespective of total density, with a few highly aggressive dominant individuals controlling one tank ("dominant" tank) and the majority of the fish preferring to occupy the second tank ("crowded" tank). This distribution pattern resembled an ideal despotic distribution (IDD), first described in birds, where movement between patches was controlled by intraspecific competition (Fretwell, 1972). The IDD has previously been described in laboratory situations in Salmonids, where dominant individuals excluded other individuals from a favourable patch (Hakoyama and Iguchi, 2001; Maclean et al., 2005). In our study, although behavioural quantifications of the individuals in the "dominant" tank were not carried out, observation of the fish confirmed that they displayed agonistic behaviours towards other individuals in the tank and fish attempting to enter the tank. Furthermore, it was observed that with increasing density, apart from the few dominant aggressive individuals occupying the "dominant" tank, there was a spillover of individuals from the "crowded" tank entering the "dominant" tank that did not perform aggressive acts. These individuals stayed immobile and accumulated in the "dominant" tank close to the doorway between the two tanks, a behaviour which is typically observed in subordinate fish (Abbott et al., 1985; Øverli et al., 1999; Winberg and Nilsson, 1993; Øverli et al., 1998). This distribution pattern was especially distinct in the two-tank system stocked with the highest total density, with the "dominant" tank occupied by a few dominant aggressive individuals and a gradual accumulation of subordinate individuals. The results indicated a negative relationship between the percentage of fish in the "crowded" tank and the total density stocked. Specifically, the percentage of fish in the "crowded" tank decreased significantly with increasing total stocking density (20, 40 and 80 kg m^{-3}). As a result of this distribution pattern in the tanks, irrespective of the time of day (sampling time, "dark" or "light"), the mean absolute density in the "crowded" tank stocked at a total density of 20 kg m^{-3} was 33 kg m^{-3}, at 40 kg m^{-3} was 64 kg m^{-3}, and 80 kg m^{-3} was 127 kg m^{-3}. Moreover, although not significantly different, the spatial distribution was observed to be more unequal during the hours when it was light (evening sampling) than during the hours when it was dark (morning sampling). During the day the fish were provided with a food resource to compete for, resulting in a few individuals monopolising this resource in one tank ("dominant" tank) and driving out the majority of the individuals into the second tank ("crowded" tank).

Neuroendocrine indicators of stress were examined to support our behavioural observations. Interestingly, the significantly higher serotonergic activity found in the brain stem and telencephalon of the individuals in the "crowded" tank under light conditions, irrespective of density, indicated higher stress levels in these fish. This suggests that stronger social competition in the "dominant" tank during the day led to greater inequality in the observed distribution of the fish in the two-tank systems which resulted in higher stress levels in the "crowded" tank. Furthermore, we observed elevated serotonergic activity, as 5-HIAA concentrations and 5-HIAA/5-HT ratios, in the brain stem and a tendency for elevated levels in the telencephalon of the individuals in the “crowded” tank of the system stocked at the highest density (80 kg m^{-3}). Previous studies investigating social behaviour in pairs or small groups of fish found an elevation in serotonergic activity levels in individuals exposed to prolonged periods of social stress (socially subordinate individuals) (Øverli et al., 1999; Winberg et al., 1991, 1992; Winberg and Nilsson, 1993), as indicated by elevated concentrations of 5-HIAA and 5-HIAA/5-HT ratios (Winberg and Nilsson, 1993; Winberg and Lepage, 1998). Often in parallel to this is an elevation in plasma cortisol concentration, suggesting a stimulatory role of 5-HT activity on the HPI axis (Øverli et al., 1999). However, this relationship tends to weaken during prolonged stress, where HPI axis reactivity decreases while 5-HT activity remains high (Winberg and Lepage, 1998). Indeed, the plasma cortisol levels found in the individuals in the “crowded” tank of the two-tank systems in the present study were generally low, and did not co-vary with serotonergic activity. Nevertheless, these findings are not uncommon in Salmonids. Basal levels of plasma cortisol in unstressed fish below 5 ng ml^{-1}, usually between 1 and 2 ng ml^{-1}, have been found, and in chronically stressed individuals, below 10 ng ml^{-1} (Pickering and Stewart, 1984; Pickering and Pottinger, 1989). In some cases, when subjected to chronic stress, plasma cortisol levels (10 ng ml^{-1}) eventually returned to basal levels (ng ml^{-1}) after a period of time, despite the continued presence of stress (Barton et al., 1980; Pickering, 1992; Strange and Schreck, 1978). Hence, in the present study, elevated levels of serotonergic activity and low concentrations of cortisol in the “crowded” tank of the two-tank system stocked at 80 kg m^{-3} should reflect chronic stress in a crowded situation.

The positive relationship between density and fish weight suggested that larger fish accepted to be at a higher density than smaller individuals. The negative relationship between fish weight and serotonergic activity in the brain stem suggests that of the fish that have accepted to stay in the “crowded” tank, the smaller fish had higher stress levels compared to larger fish. Additional studies are needed to assess how fish size influences the distribution of fish, but our results indicate that fish size is an important factor to consider when investigating critical stocking densities. Furthermore, although water quality parameters were checked at a system level daily, they were not measured specifically at the tank level. It may be speculated that as there was such a high number of fish in the “crowded” tank of the system stocked at the highest density, the water quality may have been influenced. As a result, we cannot exclude the fact that the density effects observed on the neuroendocrine stress levels could be, in part, influenced by water quality. Therefore, additional studies are necessary to exclude the influence of this factor.

5. Conclusion

Here we have presented a method using two-tank systems to determine a level of crowding that showed signs of aversiveness in farmed rainbow trout. A negative relationship between stocking density and the percentage of fish occupying the “crowded” tank was observed. Furthermore, the neuroendocrine indicators of stress suggested the presence of chronic stress in the fish of the two-tank system stocked at the highest density (80 kg m^{-3}), with low concentrations of plasma cortisol but elevated levels of serotonergic activity found in the brain stem of the individuals in the “crowded” tank of this system. Overall, these results indicated that a level of aversiveness to crowding had been reached at the highest total density stocked, where the mean absolute density that was observed in the “crowded” tank was 126.5 ± 3.7 kg m^{-3}. A follow up study is necessary to assess if being held at the densities accepted by the fish in the present study has an impact on indicators of welfare and performance in farmed rainbow trout.

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Appendix II

Paper II: High oxygen consumption rates and scale loss indicate elevated aggressive behavior at low rearing density, while elevated serotonergic activity suggests chronic stress at high rearing densities in farmed rainbow trout

High oxygen consumption rates and scale loss indicate elevated aggressive behavior at low rearing density, while elevated serotonergic activity suggests chronic stress at high rearing densities in farmed rainbow trout

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Abstract

The effect of stocking density on indicators of welfare has been investigated by several studies on farmed rainbow trout *Oncorhynchus mykiss*. However, the densities at which indicators of welfare are compromised remain ambiguous. Here three different stocking density treatments were selected based on levels of crowding previously chosen by fish in two-tank systems. An un-crowded low density of 25 kg m^{-3}, the highest density accepted by the fish without showing indications of crowding stress of 80 kg m^{-3} as the intermediate density, and the highest density accepted by the fish showing indications of crowding stress of 140 kg m^{-3} as the high density were investigated. The aim of the present study was to examine the effect of being held at these densities on indicators of welfare. This was achieved oxygen through; oxygen consumption measurements using automated respirometry, recording fin erosion, determining scale loss and analysing plasma cortisol and brain serotonergic activity levels. The results obtained in the present study indicated that at the lowest density the fish had the space and opportunity to display their natural aggressive behaviour and that the fish held at the highest density were exposed to a situation of confinement.

Key words:

Rearing density, metabolic rate, physical injury, cortisol, serotonergic activity

1. Introduction

A number of studies have been published examining the relationship between stocking density and indicators of welfare in rainbow trout *Oncorhynchus mykiss* [1-5]. Being held at low or high stocking density has been demonstrated to potentially impact indicators of welfare in a negative manner [1,3]. Yet, concrete minimum and maximum density levels where welfare indicators are affected continue to be undefined.

Of all the studies to date, a wide range of stocking densities have been investigated [1-5]. However, the majority of these studies lack details about how the densities that were investigated were selected. It may be speculated that previous research and the densities observed in practice influenced these decisions. The present study is unique in this regard, as the densities examined here were reflective of levels of crowding established in a study by Laursen et al. [6]. In the aforementioned study, the spatial distribution of fish held at different densities in two tank systems was used to determine a level of aversion to crowding [6]. The aim of the present study was to determine if being held at the chosen densities had an influence on indicators of welfare.

Previous research has found that oxygen consumption rates are elevated in fish held at higher stocking density [7,8]. Stress has been attributed as the cause of this increase [8]. Elevated oxygen consumption rates may also reflect high levels of behavioural activity, such as aggressive behaviour and social hierarchy formations [9-12], which is often observed in fish held at low stocking density [1,12]. Therefore, oxygen consumption rates could provide information regarding stress levels at different densities. Furthermore, it could give considerable insight into the behavioural activity levels and social dynamics at different densities. This is especially relevant as aggressive behaviour has been highlighted as a welfare issue in aquaculture [1].

Physical injuries may be considered a welfare concern, based on the five freedoms framework [1,13]. Injury, in the form of fin erosion, is a commonly observed condition in farmed rainbow trout and has been linked to high stocking density [1,3,14]. The condition is thought to be caused by several factors, such as; aggressive encounters with conspecifics [15], abrasion and collision with the rearing environment, water quality, infection and stress [1,16]. As higher stocking density can enhance the occurrence of these factors, it is thought to contribute to this condition [1]. Furthermore, injury caused by excessive scale loss could have consequences for welfare. Injury, quantified as scale loss, due to fighting has previously been investigated in cichlids (*Tilapia zillii*) [17]. During aggressive encounters in rainbow trout, individuals are observed to lose a large quantity of scales. Furthermore, abrasion with the environment has been proposed to contribute to this loss [1,16]. To our knowledge, scale loss has not been investigated before as an indicator of welfare, especially in relation to stocking density. In the present study, physical injury, as indicated by fin erosion and scale loss, could give further insight into the level of social interaction within the tank in relation to stocking density.

Measures of the physiological stress response have been a focus in previous studies. Chronic activation of the stress response, specifically cortisol, can have deleterious consequences for growth, disease resistance, and reproduction, thereby providing indications for compromised welfare in individuals [18]. Although the influence of stocking density on cortisol levels has given contrasting results [1], it continues to be a valuable indicator of stress in fish. Furthermore, serotonergic activity, the ratio between the brain tissue concentration of serotonin (5-HT, monoamine) and 5-hydroxyindoleacetic acid (5-HIAA, metabolite), has previously been used as an indicator of chronic social stress in salmonid fish held in pairs and small groups [19-22]. Recently, it has been used as an indicator of stress in relation to stocking density in rainbow trout [2]. Furthermore, in the study by Laursen et al. [6], elevated serotonergic activity levels were found in individuals held in crowded conditions.

In summary, here three densities were selected based on levels of crowding chosen by the fish using a method by two-tank systems [6]; an un-crowded low density of 25 kg m^{-3}, the highest density accepted by the fish without showing indications of crowding stress of 80 kg m^{-3}, and the highest density accepted by the fish showing indications of crowding stress of 140 kg m^{-3}. The aim of the present study was to investigate the influence of being held at these densities on indicators of welfare; oxygen consumption rates, physical condition and neuroendocrine indicators of stress.

2. Materials and Methods

2.1 Experimental fish

Rainbow trout from Store Restrup fish farm, Denmark were used for the present study. The fish were transported by truck to the Danish Technical University, Institute of Aquatic Resources (DTU Aqua) in Hirtshals and upon arrival stocked directly into quarantine tanks, as a preventative measure against disease and parasites. While in quarantine, the fish were fed at 0.75 % of the total biomass in the tank per day. Additionally, the salt content in the water was slowly increased to 15 ‰ while the fish were held in quarantine, and decreased again before the fish were moved to the experimental facility. The fish were held in quarantine conditions for a period of 15 days, after which they were available to be used for experiments. At the time of delivery, the fish had an average individual weight of 130 grams. At the time the fish were used for the experiment, they had an average weight of 170 ± 2.3 grams.

2.2 Experimental facilities

The experiment was carried out using the twelve tank experimental facility previously detailed by Larsen et al. [8] and McKenzie et al. [2] and is briefly outlined here. The fish were held in nine of the white circular tanks. Each tank was one meter in height and diameter, and held a volume of 600 liters.

All the tanks were supplied with water from the same recirculating biofilter system. Water from the system was pumped to the inflow of each tank and entered the tank through a vertical inlet pipe (20 mm diameter, 70 cm length), fixed to the wall of the tank. Small holes (4 mm diameter) along the length of the inlet pipe created pressure to the water flowing into the tank, thereby circulating the water around the tank. A circular column (35 cm diameter) standing at the center of the tank aided the circular flow of the water in the tank. The speed of the water current could be adjusted by increasing or decreasing the amount of water entering the inlet pipe. The water left the tank through a drain at the center bottom of the tank and passed through a whirl separator, next to the tank, before returning to the biofilter. Solid waste; faeces, uneaten pellets and fish scales were collected in the whirl separator. A valve at the bottom of the whirl separator could be opened to allow for removal of the collected solid waste.

A constant flow of oxygen was supplied to each tank by a diffuser at the inflow of each tank, providing the baseline oxygen level desired for the tank. Each tank was provided with an electrode (Oxyguard standard probe), which measured the oxygen concentrations continuously. The electrode in each tank was connected to a transmitter, where the desired oxygen concentration could be set for the tank. Whenever the oxygen concentration in the tank fell below the desired level, a boost of oxygen was released into the tank until the concentration in the tank again reached the desired level. The lowest oxygen concentration acceptable could be set for each tank at the transmitter. In the present study, the transmitter was programmed so that if the oxygen concentration in a tank fell below 60 % saturation (5.5 mg L^{-1}), an emergency supply of oxygen from an alternative oxygen source was started. The data from the transmitters was saved onto a data logger, for later analysis (see section 2.5.1.).

The experimental tanks were modified to function as respirometers. Each tank was fitted with a three-way valve at the inflow to the tank. This valve was open under normal circumstances, allowing oxygen and aerated water from the system to be pumped into the tank. The valve could be closed, cutting off the oxygen and fresh water supply from the system, thereby circulating the existing water in the tank. During this period, the decline in the oxygen concentration in the tank was measured automatically and registered on the data logger. The three-way valve was connected to a digital timer, which could be programmed to close the three-valve for a pre-determined interval.

2.3 Experimental protocol

Three stocking densities were investigated during the experiment. The densities selected reflected the results of a study by Laursen et al. [6], where two-tank systems were used as a method to determine a level of crowding experienced as aversive by the fish. In that study, behavioural and neuroendocrine measures were used as indicators of crowding stress. Here, a density of 25 kg m^{-3} served as an un-crowded low density (LD), the highest density accepted by the fish without showing indications of crowding stress of 80 kg m^{-3} as the intermediate density (ID), and the highest density accepted by the fish showing indications of crowding stress of 140 kg m^{-3} as the high density (HD).

The fish were transported from the quarantine facilities to the experimental facilities to be stocked randomly into the experimental tanks; at 25 kg m^{-3}, 80 kg m^{-3} and 140 kg m^{-3} in triplicate. A subsample of 30 individuals for each tank were lightly anaesthetized (Ethylene glycol monophenyl ether) and pit tagged and adipose fin clipped for individual identification throughout the experiment. This was done at initial stocking, to allow for a period of recovery of the fish after the procedure. Subsequently, the sub-sample of 30 individuals was added to each tank and the remaining biomass was added to each tank to achieve the desired density. The sub samples of 30 fish from each tank were individually weighed, measured for fork length and checked for fin damage at each subsequent weighing session.

After stocking, the fish were acclimated in the experimental tanks for a period of approximately two weeks where the feeding level was gradually increased to 1.5% of the total tank biomass per day. This was done to allow the biofilter of the recirculation system to cope with the biomass of fish and to ensure that the water quality parameters were adequate for the fish. Furthermore, it allowed time for making adjustments to the oxygen levels according to the densities in each tank for oxygen consumption measurements. At the end of the acclimation period, the biomass in each tank was determined and re-adjusted to the desired density by removing excess kilograms. The number of fish in the tank was counted.

The experimental duration was 28 days, consisting of two growth periods of 12 days. During each growth period, the fish were fed for 12 days, whereafter the biomass in each tank was weighed. Prior to weighing, the fish were given a period of fasting for a day to minimize the risk for infection after weighing.

Each tank was fed at 1.5 % of the estimated tank biomass per day, with 3 mm pellets (EFICO Enviro 920, BioMar A/S). The fish were fed in the morning at 09:00 with automatic belt feeders for a period of six hours. In the afternoon after feeding, the solid waste was collected from the whirl separator and the numbers of uneaten pellets were counted to determine feed waste. Additionally, the scales lost by the fish were also separated out from the solid waste and collected for later weighing.

On the day of fasting, to determine basal stress levels, a subsample of four individuals from each tank (un-pit tagged) were sacrificed during daylight hours and blood and brain samples were collected from these fish. The blood samples were for later analysis of plasma cortisol concentration and the brain samples for later analysis of brain monoamine and metabolites.

On the day of weighing, the total biomass in each tank was recorded. The 30 pit tagged individuals were separated from the biomass, and individually weighed, measured for fork length and checked for fin erosion. They were then re-stocked into the tank, and the remaining biomass was added to the tank to achieve the desired density. The excess kilograms were discarded. This process was repeated for the second experimental period.

Oxygen consumption was measured continuously throughout the growth period. Measurements were started on the first day of feeding and stopped on the day of weighing. Oxygen levels were set at 80% (8.5 mg L^{-1}) in the tanks held at the low density, 110% (11.5 mg L^{-1}) at the intermediate density, and 120% (12 mg L^{-1}) at the high density. Oxygen concentrations were set at these levels for practical reasons, to obtain a long enough closing period to be able to measure oxygen consumption and to ensure that oxygen levels did not fall below the critical level (60%, 5.5 mg L^{-1}) at the intermediate and high densities during the oxygen consumption measurement period.

Water quality parameters; nitrite (NO_2^-), nitrate (NO_3^-), ammonia (NH_3/NH_4^+), pH and temperature, were measured daily at the system level to ensure that they were within optimal levels for the fish. The temperature of the water in the system was controlled at 16 °C. A slow water current of approximately 0.5 body lengths per second was provided to each tank to even out the distribution of the fish and the pellets in the tank. Light conditions were at 14.5 light and 9.5 dark hours, with the lights automatically switching on at 07:30 and switching off at 22:00.

2.4 Measurements

2.4.1. Oxygen consumption

Oxygen consumption measurements were taken continuously using the automated respirometry system. Ever hour, the three-way valve at the inflow to the tank would close and thereby shut off the oxygen supply to each tank. The valve remained closed for a period of 8 minutes during the day time hours (09:00 – 17:00) and 6 minutes during the night times hours (18:00 – 08:00). The oxygen concentrations in the tanks were registered from the transmitters to the data logger every 20 seconds during the period when the valve was closed.

Oxygen consumption was calculated as previously described by Larsen et al. [8]. For each hourly measurement period on each experimental day, the decline in the oxygen concentration in each tank was used to perform a linear regression. In the present study, the data from the last 5 minutes of the measurement period and the last 3 minutes of the measurement period were used from the day time hours and night time hours respectively. The biomass in each tank was estimated for each day using the specific growth rate (SGR). The absolute volume of water in the tank was obtained by subtracting the estimated biomass on the day from the known volume of water the tank could hold. The slope value obtained from the linear regression, the estimated total biomass of fish in the tank on the day and the total volume of water in the tank on the day were used to calculate the oxygen consumed by the fish, as milligrams of oxygen consumed per kilogram of fish per hour (mg O_2 kg $^{-1}$ hr $^{-1}$).

To ensure direct comparability of the concentrations of oxygen consumed between the tanks, the data used was selected from the days where the body mass increase of the fish was similar in each of the tanks. In the present study, a period of days from when the fish grew from 190 to 220 grams (mean body weight 205 grams) was chosen. The data on the amount of oxygen consumed taken from those days was corrected to a 205 gram body weight fish, using the method detailed by Larsen et al. [7].

The hourly oxygen consumption rates during a daily cycle were determined for the three density treatments. The minimum, median and maximum oxygen consumption rates were determined from the daily cycle for comparison between the three density treatments. Generally, the minimum amount of oxygen was consumed between the hours of 06:00 and 08:00 and the maximum between the hours of 11:00 and 13:00. The median rates of oxygen consumption were between the time of 21:00 and 23:00. Furthermore, the total amount of oxygen consumed during the selected days (outlined above) was determined for the three density treatments.

2.4.2. Physical indicators

Fish scales were collected from each of the tanks daily. After feeding was finished in the afternoon, the solid waste from the tank that had collected in the whirl separator was flushed out and collected in a bucket. The contents of the bucket were emptied into a sieve where the scales were separated from the faeces and uneaten pellets. The scales were collected in plastic containers for later weighing.

The scales were dried and weighed on a pre-weighed filter paper (Qualitative filter paper, 413; WWR). Before weighing, the filter paper with the scales was put in the dryer at 60 °C for a period of one hour. To determine the grams of scales lost per kilogram fish (g kg^{-1}), the weight of the scales was divided by the estimated biomass in the tank (using the SGR). The total amount of scale loss was determined for each density treatments for the same period as for the total oxygen consumed, the selected days from when the fish grew from 190 grams to 220 grams.

Fin erosion was determined from the subsample of 30 pit tagged individuals from each tank, using the photographic key developed by Hoyle et al. [12]. During sampling, the individuals were lightly anaesthetized and examined. Each fin type per individual was compared to the pre-developed photographic key and given a score from one to five. A score of one was considered to be a fin in good condition and five a fin showing considerable damage.

2.4.3. Neuroendrocrine indicators of stress

A sub sample of four individuals per tank were sacrificed by an overdose of anaesthetic (Ethylene glycol monophenyl ether). Blood samples were collected from the caudal vein using 1 ml syringes. The blood samples were centrifuged at 15,000 rpm for 5 minutes and the plasma was separated into 1 ml eppendorf tubes and frozen at -80 °C for later analysis. During analysis, cortisol was extracted from the plasma by mixing with ethyl ether. The solvent was evaporated using a vacuum centrifuge and the remaining residue was re-suspended in an extraction buffer (ELISA kit extraction buffer). Cortisol concentrations (ng ml^{-1}) were quantified using the ELISA kit standard method (Neogen, Product #402710).

Whole brains were dissected out from each fish and separated into four parts; the brain stem, hypothalamus, telencephalon and optic lobes. Each brain part was frozen separately at -80 °C for later analysis. Before analysis, each frozen brain part was individually weighed. After weighing, the brain part was homogenised in a homogenising reagent (4% perchloric acid, 0.2% Ethylenediaminetetraacetic acid, 40 ng ml^{-1} dihydroxi benzylamine hydroxide solution). The solvent was then centrifuged at 10,000 rpm at 4 °C for 10 minutes. The supernatant was assayed by High Performance Liquid Chromatography (HPLC) with electrochemical detection to quantify the concentration of 5-HT (serotonin) and its catabolite 5-Hydroxyindoleacetic acid (5-HIAA). The HPLC system consisted of a mobile phase (buffer solution; 10.35 g l^{-1} sodium phosphate, 0.3252 g l^{-1} sodium octyl sulphate, 0.0037 g l^{-1} EDTA, 7% acetonitril in deionised water), a solvent delivery system (Shimadzu, LC-10AD), an auto injector (Famos, Spark), a reverse phase column (4.6 mm 100 mm, Hichrom, C18, 3.5 mm) and an ESA Coulochem II detector (ESA, Bedford, MA, USA) with two electrodes at -40 mV and +320 mV. A conditioning electrode with a potential of +40 mV is used to oxidize possible contaminants before analysis. Brain 5-HT and 5-HIAA were quantified by comparing them with standard solutions of known concentrations and corrected for recovery of the internal standard using HPLC software (CSW, DataApex Ltd, Czech Republic).

2.5 Statistical analyses

Oxygen consumption rates were analysed using one-way ANOVA's, where density was the independent variable and the dependent variable either the minimum, median, maximum or total oxygen consumption concentration. A tukey's post hoc test was carried to determine where the significances were present. A one-way ANOVA was used to analyse the differences between density treatment on the total amount of scale loss. A Spearman rank test was done to analyse the relationship between the total scale loss and total oxygen consumption. Fin scores were analysed using a Kruskal-Wallis by ranks (comparing multiple independent variables) test, where density was the independent variable and each fin type the dependent variable. A two-way ANOVA was performed to determine if there was a difference in plasma cortisol concentrations between density treatments (LD, ID & HD) and experimental period (1 &2). Density treatment and experimental period were the independent variable and log concentrations of plasma cortisol the dependent variable. Furthermore, a two-way ANOVA was performed to determine if there was a difference in the arcsin ratio of 5-HIAA/5-HT between density treatment and experimental period. The difference between density treatment in the log concentrations of 5-HIAA and 5-HT were determined using a one-way ANOVA. A Tukey post hoc test was done to determine where the significances occurred. All statistical analyses were carried out using the computer program Statistica (version 11). The values presented in the figures are mean ± standard error.

3. Results

3.1. Oxygen consumption

The mean daily pattern of mass specific oxygen consumption rates for the three density treatments is shown in Figure 1. The general pattern at all densities is described as follows: consumption rates were lowest from midnight (00:00) until 09:00, the minimum rates being between the hours of 06:00 to 08:00. At 08:00, after the lights automatically turned on at 07:30 and the fish anticipated being fed, there was a slight increase in consumption until 09:00, where after there was a sharp increase. After reaching maximum rates at around mid day (11:00 to 13:00), consumption rates started to decrease rapidly until the hour of 17:00. Thereafter, rates decreased slowly throughout the night. It was observed that in the low density tanks, the pattern was erratic compared to the smooth pattern observed in the tanks at the higher densities.

The average minimum (time 06:00 – 08:00; Fig. 2), median (time 21:00 – 23:00; Fig. 2), and maximum (time 11:00 – 13:00) oxygen consumption rates were significantly different between the density treatments ($p<0.001$, $p<0.001$, $p<0.001$ respectively; Fig. 2), with the amount of oxygen consumed decreasing with increasing density (Fig. 2). Specifically, at minimum rates, there was a difference between the LD and ID ($p<0.001$), between LD and HD ($p<0.001$), and between ID and HD ($p<0.001$). At median and maximum rates, a similar pattern was observed, with a difference between the densities of LD and ID ($p=0.003$, $p<0.001$ respectively), between LD and HD ($p<0.001$, $p<0.001$ respectively), and between ID and HD ($p=0.017$, $p<0.001$ respectively). This pattern was also reflected in the total oxygen consumed during the selected days, with the rates decreasing with increasing density ($p=0.010$, Fig. 3). Specifically, there was a difference between the LD and HD ($p=0.009$), but not between the LD and ID ($p=0.052$) or between the ID and HD ($p=0.325$).

3.2. Scale loss

The total scale loss differed significantly between the density treatments (p=0.006; Fig. 4), where the amount of scales lost per kg of fish decreased with increasing density. Specifically, there was a difference between the LD and ID (p=0.025), between LD and HD (p=0.006), but not between the ID and HD (p=0.432). Furthermore, there was a positive correlation between total scale loss (g kg^{-1}) and total oxygen consumption (mg O_2 kg^{-1}; R^2=0.917, p<0.001; Fig. 5).

3.3. Fin scores

Of the different fin types, there was a significant difference between the density treatments in the damage to the caudal fin (p=0.012; Fig. 6). Specifically, there was a difference between the LD and ID (p=0.016), but not between LD and HD (p=0.450) or the ID and HD (0.553). There was also a significant difference between density treatments in the damage to the left pelvic fin (0.030; Fig. 6). However, no difference was found when looking specifically between densities; LD and ID (p=0.481), LD and HD (p=0.073) or ID and HD (p=1.000).

3.4. Neuroendocrine indicators

3.4.1. Plasma cortisol

The plasma cortisol concentrations showed no significant differences between the three density treatments (LD, ID & HD), despite a slight elevation in the levels at the highest density stocked (p=0.284; Fig. 7). There was no difference between experimental period (1 & 2; p=0.265), or interaction between density treatment and experimental period (p=0.594).

3.4.2. Serotonergic activity (5-HIAA/5-HT)

The serotonergic activity levels in the brain stem of the individuals held at LD were lower compared to in the individuals held at the higher densities (p<0.001; Fig. 8A). Specifically, there was a difference between the LD and ID (p<0.001), between LD and HD (p<0.001), and between the ID and HD (p=0.045). Significantly higher levels of serotonergic activity was found during the first experimental period compared to the second (p<0.001). There was also an interaction between density treatment and experimental period (p=0.016). Furthermore, the concentration of 5-HIAA in the brain stem increased with increasing density (p<0.001; Table 1). There were no differences in serotonergic activity levels in the telencephalon or hypothalamus between the density treatments (p=0.594, p=0.495 respectively; Fig. 8B & 8C). However, the concentration of 5-HIAA in the telencephalon was elevated at the HD compared to the ID and LD (p=0.006; Table 1). Furthermore, significantly higher levels of serotonergic activity was found during the first experimental period compared to the second in both the telencephalon (p=0.002) and hypothalamus (p=0.006).

4. Discussion

A negative relationship between oxygen consumption and stocking density was observed in the present study. This is in contrast to previous results in rainbow trout, where high stocking density was associated with elevated metabolic rates compared to low stocking density [2,8]. At higher densities, fish are exposed to crowding and the physiological disturbance caused by chronic stress can result in increased oxygen uptake and usage [7,23]. Behavioural aspects may also explain increased metabolic rates in fish. Bursts of spontaneous activity, such as agonistic interactions, may result in elevated metabolic rates [9,10]. Indeed, high levels of spontaneous activity associated with agonistic behavior have been shown to cause elevated metabolic rates in juvenile sockeye salmon and rainbow trout [9,12]. The elevated oxygen consumption rates found in the tanks held at the lowest density compared to the highest density in the current study may be reflective of high levels of spontaneous aggressive behaviour. The hourly oxygen consumption rates at the lowest density showed a more erratic pattern compared to in the tanks stocked at the higher densities, where a smoother hourly pattern was observed.

Additionally to oxygen consumption, a negative relationship between scale loss and stocking density was observed, with a decrease in total scale loss with increasing density. Scale loss has been associated with abrasion with the environment [16]. It could also be the result of aggressive interactions between conspecifics, as has been observed during fights in cichlids [17] and rainbow trout. During a fight between a pair of fish, individuals will violently attack, nip and bite each other [15,22], which results in visible scaring and a large quantity of scale loss. As scale loss was highest at the lowest density in the present study where the fish had abundant space and lowest at the highest density where the fish were crowded together, it is unlikely that scale loss was due to passive abrasion with the environment but rather due to aggressive interactions between individuals. Indeed, aggressive interactions have been observed to increase in rainbow trout with decreasing stocking density [1,12]. Interestingly, the positive correlation between scale loss and oxygen consumption rates further strengthens the implications for the occurrence of natural aggressive behaviour of the fish held in the tanks at the lowest density and that this behaviour was diminished with increasing density.

Furthermore, a degree of the erosion observed in certain fin types in rainbow trout has been attributed to aggressive behaviour [1,15] and could therefore be used as an indicator of such behaviour in the present study. As aggressive behaviour was concluded to occur at the lowest density, the same pattern could have been expected in fin erosion. However, in the present study this pattern was difficult to interpret; as of the different fin types, fin erosion was least visible in the caudal and left pelvic fin at the lowest density. Damage to the fins was more evident in the individuals held at the intermediate and high density. However, this may not be surprising as several studies have documented an increase in fin erosion with increasing density [1,3,14]. Poor water quality, abrasion with the environment and elevated stress levels associated with high density situations have been found to increase the severity of fin erosion [1].

The plasma cortisol concentrations did not differ between the densities. Furthermore, the values were generally low, suggesting that stress levels were reduced in the individuals held at all of the densities. However, low levels of cortisol have been observed in chronically stressed individuals in some cases [24,25]. For example, it has previously been found that cortical concentrations returned to basal levels within a week when exposed to a chronic stressor [23]. One explanation given for this observation is that a negative feedback mechanism acts on the hypothalamus causing a down regulation of cortisol [26].

Similarly to the plasma cortisol concentrations, rearing density did not affect serotonergic activity in the hypothalamus and telencephalon. On the other hand, serotonergic activity was found to be significantly higher in the brain stem. This region specificity may be related to that serotonin (5-HT) is mainly synthesised in the raphe nuclei, a structure found in this brain part [27]. Previous studies have shown a strong positive relationship between 5-HTergic activity in this brain part and cortisol during moderate to short term stress [19-22]. However, during periods of chronic stress this relationship may weaken, where serotonergic activity remains high while cortisol concentration declines [28]. Hence, in the present study, chronic stress was indicated by low concentrations of plasma cortisol and elevated serotonergic activity (5HIAA/5HT) levels. Indeed, low levels of cortisol and elevated brain stem 5-HTergic activity levels have previously been associated with chronic stress in fish held at higher densities [6]. As a result, the neuroendocrine indicators in the present study showed that stress levels were most elevated at the highest density stocked, followed by the intermediate density and lowest at the low density.

Taken together, the results discussed here give insight into to the suggestion that at the lowest density the fish had the space and opportunity to display their natural behaviour and that the fish held at the highest density were exposed to a situation of confinement. At the lowest density, the high oxygen consumption rates and high scale loss indicated increased natural behaviour levels, in the form of aggressive encounters. Additionally, the low stress levels could indicate that the behaviour displayed had a stress reducing effect. Displaced aggression is a stress reducing behavioural outlet, where aggression towards others functions as a coping strategy to reduce stress [29]. This type of behaviour has been observed in rainbow trout held in small groups [29]. At the highest density, the lower levels of oxygen consumption and scale loss suggest that due to the confined conditions the fish were unable to display natural behaviour. Furthermore, the individuals held at this density showed signs of elevated stress levels, which are indicative of a crowded environment.

5. Conclusion

The aim of the present study was to assess indicators of behaviour and welfare in fish held at three different densities; a low, intermediate and high density. The densities investigated in the present study reflect levels of crowding that were accepted by fish in a two-tank system. The results discussed here indicated that at the lowest density the fish had the space and opportunity to display their natural behaviour and that the fish held at the highest density were exposed to a situation of confinement.

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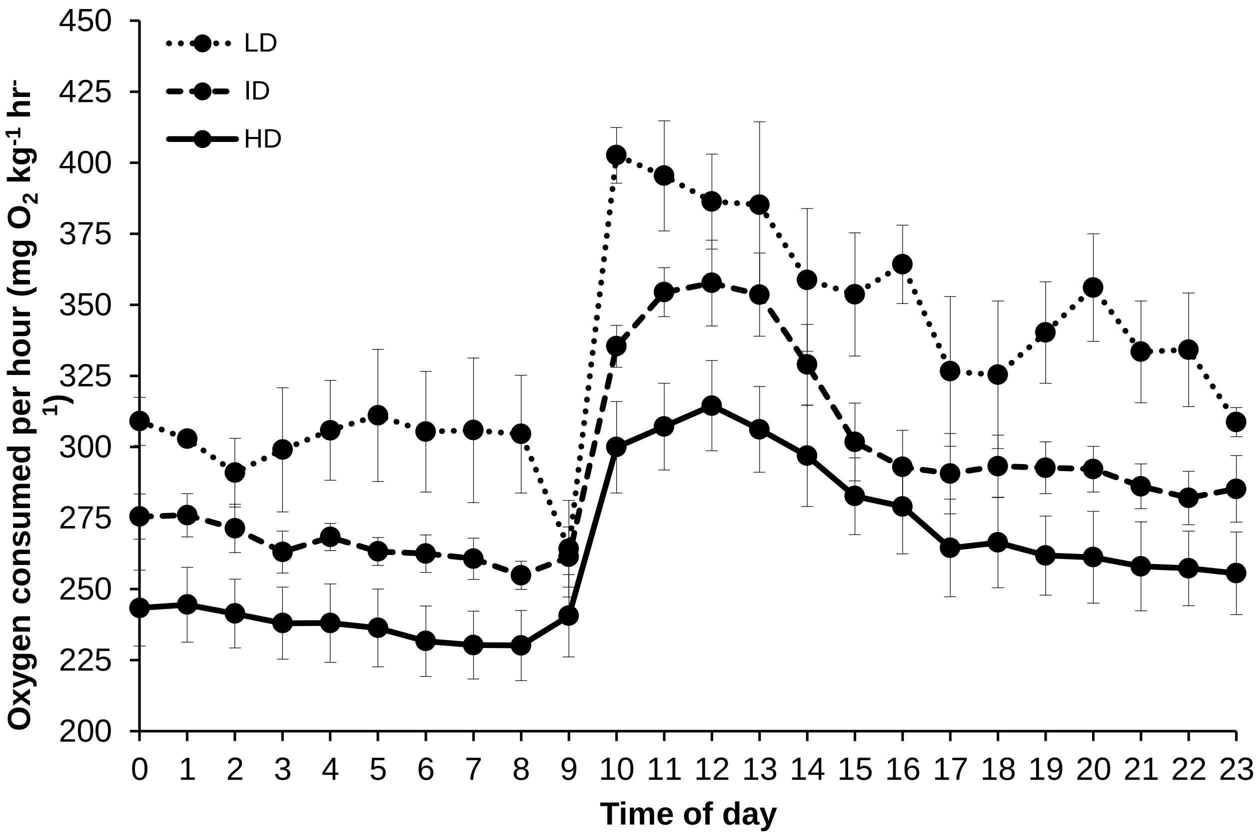


Figure 1. The hourly oxygen consumption rates (mg O_2 kg^{-1} hr^{-1}) between the three density treatments (n=3 tanks per treatment); 25 kg m^{3-1} (LD), 80 kg m^{3-1} (ID) and 140 kg m^{3-1} (HD), normalised for a 205 gram fish. The days during which the fish grew from 190 grams to 220 grams are used.

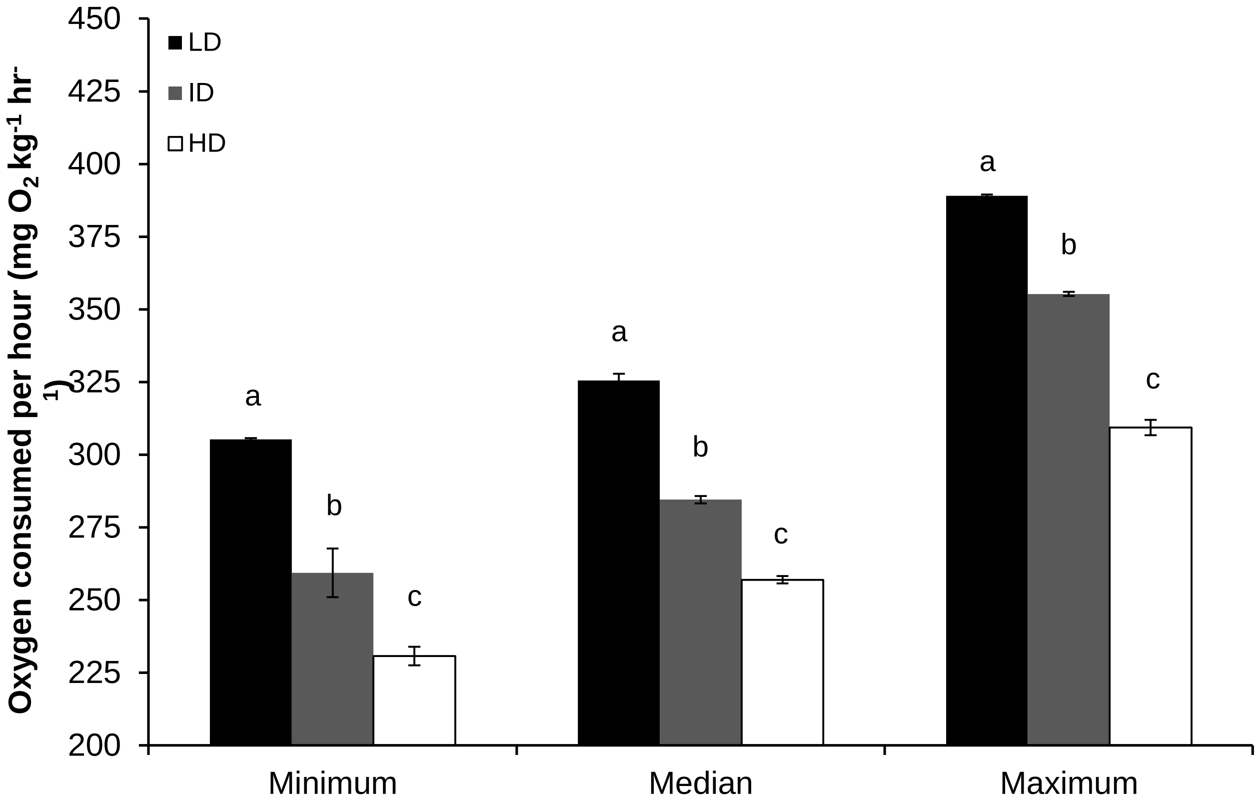


Figure 2. The minimum, median and maximum oxygen consumption rates (mg O_2 kg^{-1} hr^{-1}) during the daily cycle (Fig. 1) between the density treatments (n=3 tanks per treatment); 25 kg m^{3-1} (LD), 80 kg m^{3-1} (ID) and 140 kg m^{3-1} (HD). The minimum rates were between time of day 6 to 8, the median rates were from time of day 21 to 23, and maximum rates from time of day 11 to 13 of the daily cycle from Figure 1. The letters denote where the significances lie.

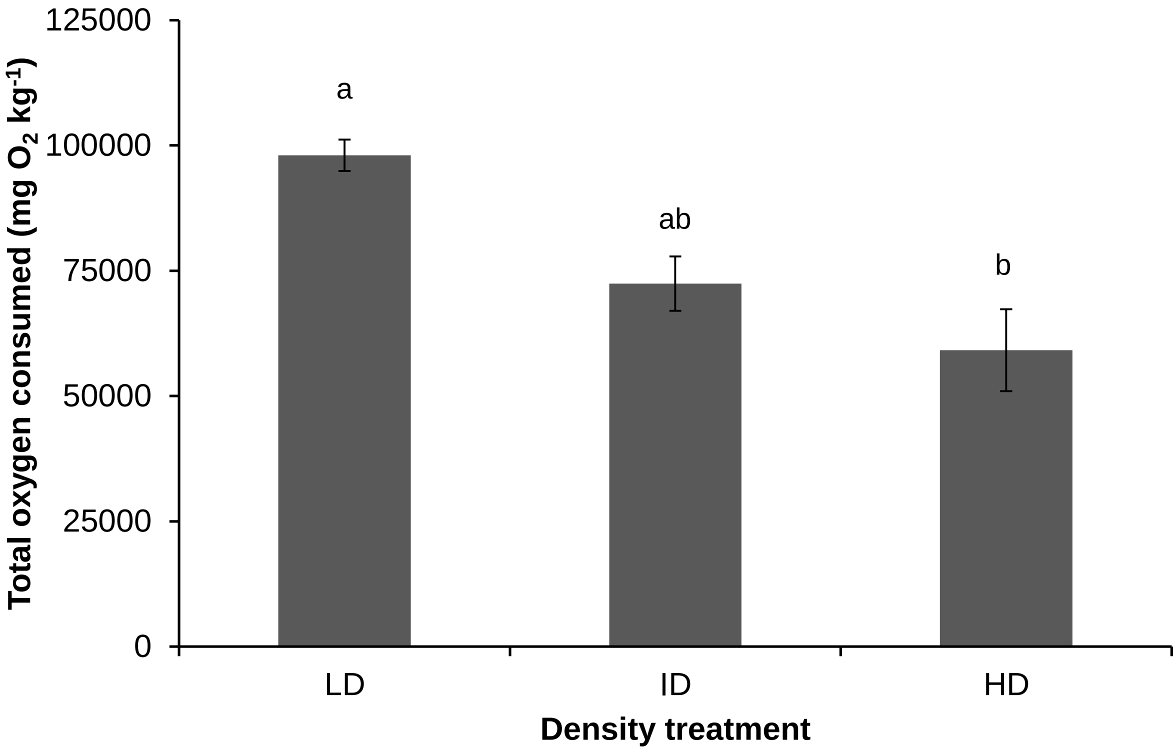


Figure 3. The total oxygen consumption rates (mg O_2 kg^{-1}) between the density treatments (n=3 tanks per treatment); 25 kg m^{3-1} (LD), 80 kg m^{3-1} (ID) and 140 kg m^{3-1} (HD) during the period (selected days) when the fish grew from from 190 grams to 220 grams. The letters denote where the significances lie.

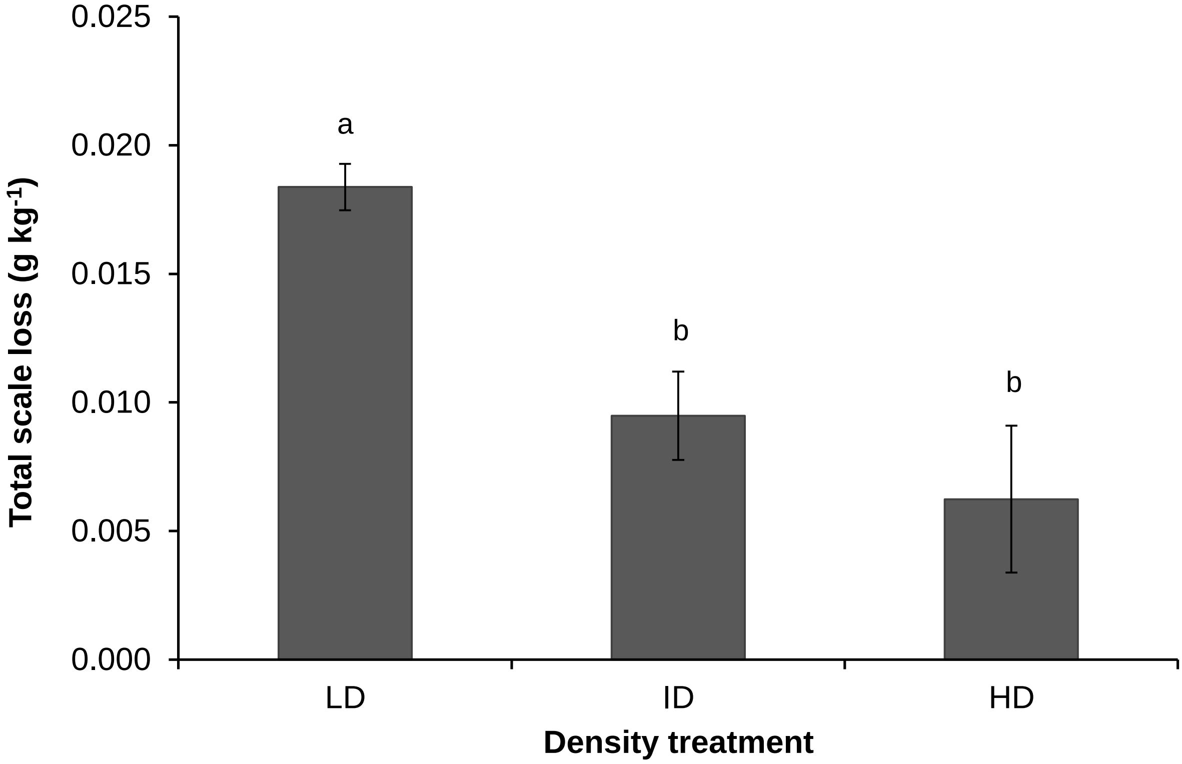


Figure 4. The total scale loss (g kg^{-1}) between the density treatments (n=3 tanks per treatment); 25 kg m^{3-1} (LD), 80 kg m^{3-1} (ID) and 140 kg m^{3-1} (HD) during the period (selected days) when the fish grew from from 190 grams to 220 grams. The letters denote where the significances lie.

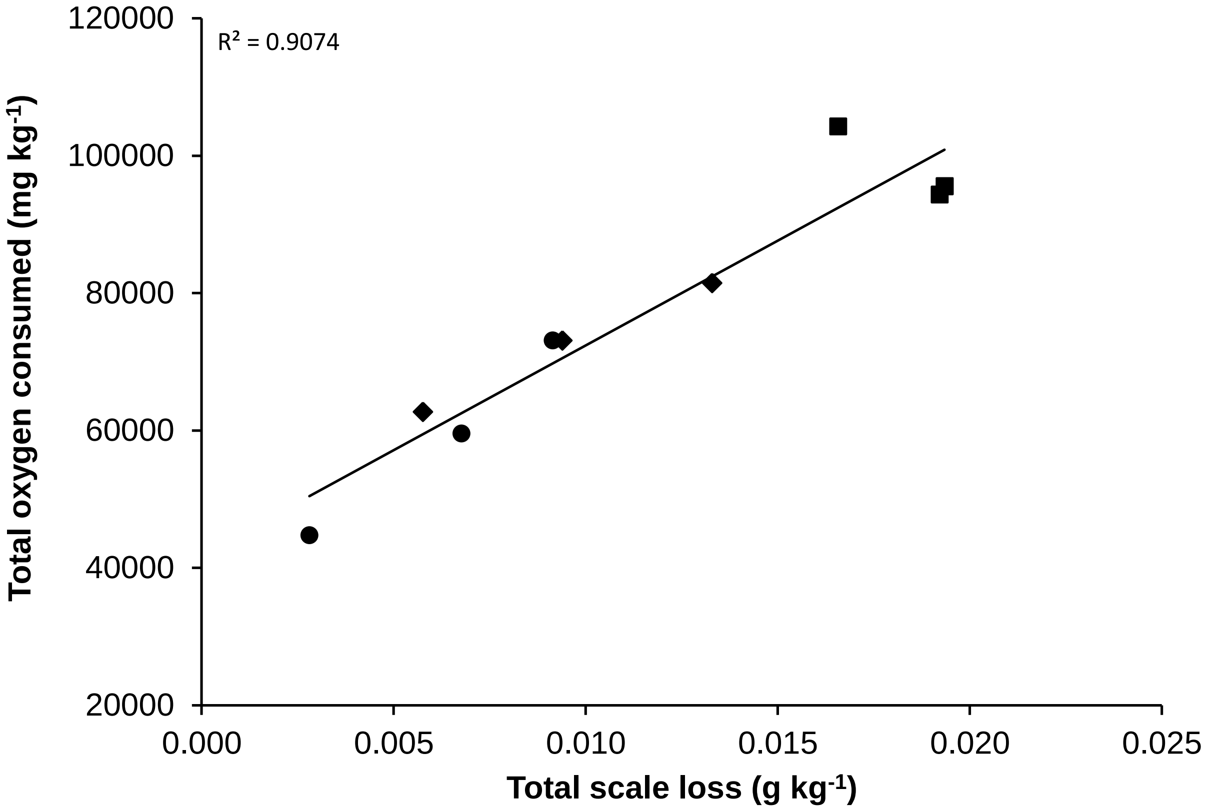


Figure 5. The relationship between the total scale loss (g kg^{-1}) and the total oxygen consumption (mg O_2 kg^{-1}) between the density treatments (n=3 tanks per treatment); 25 kg m^{3-1} (LD), 80 kg m^{3-1} (ID) and 140 kg m^{3-1} (HD) during the period (selected days) when the fish grew from from 190 grams to 220 grams. Cirlces represent the LD tanks, diamonds represent the ID tanks and squares represent HD tanks.

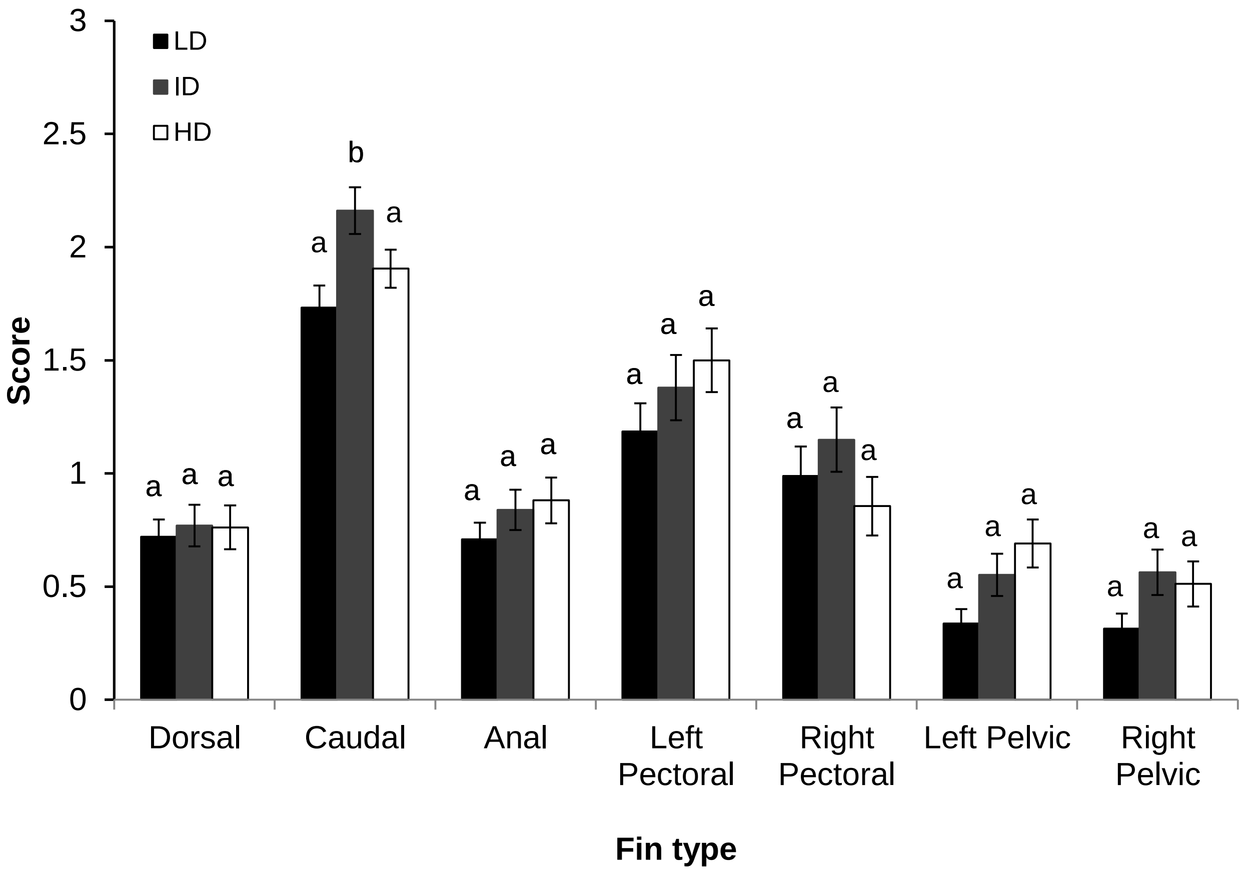


Figure 6. The fin score for each fin type of individuals (n=30 individuals per tank in triplicate) between the density treatments; 25 kg m^{3-1} (LD), 80 kg m^{3-1} (ID) and 140 kg m^{3-1} (HD) at termination of the experiment. The letters denote where the significances lie.

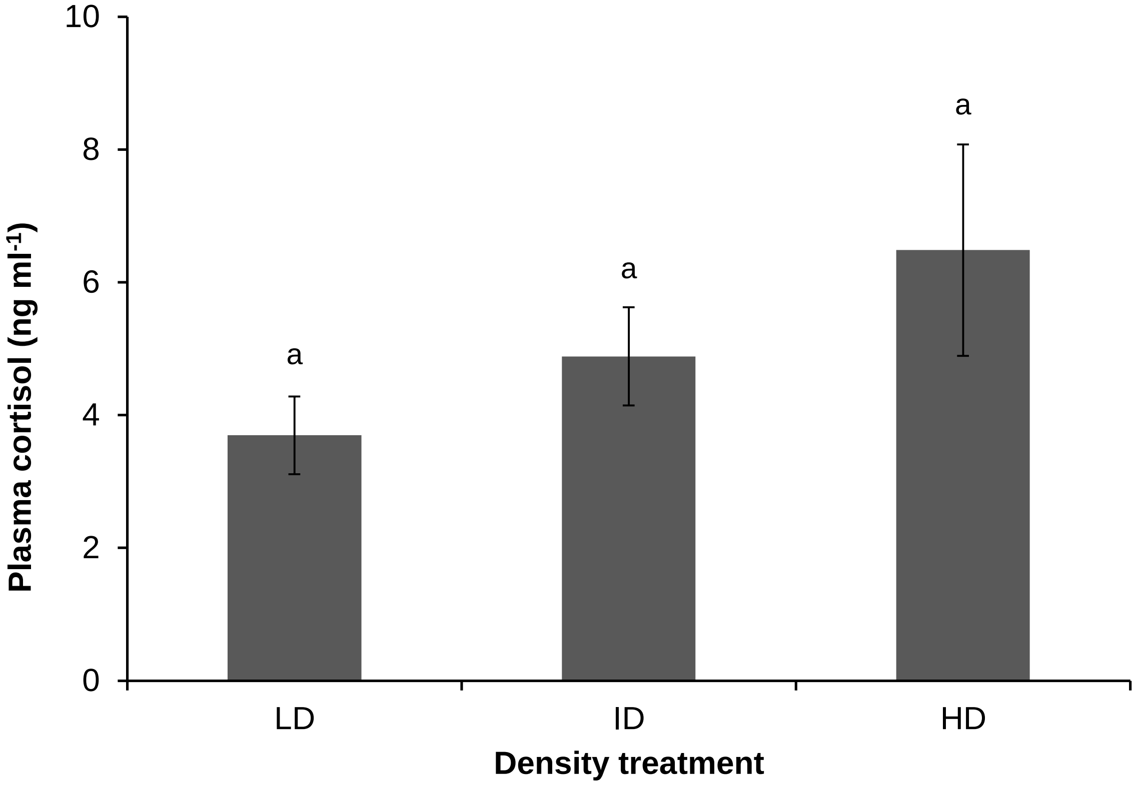
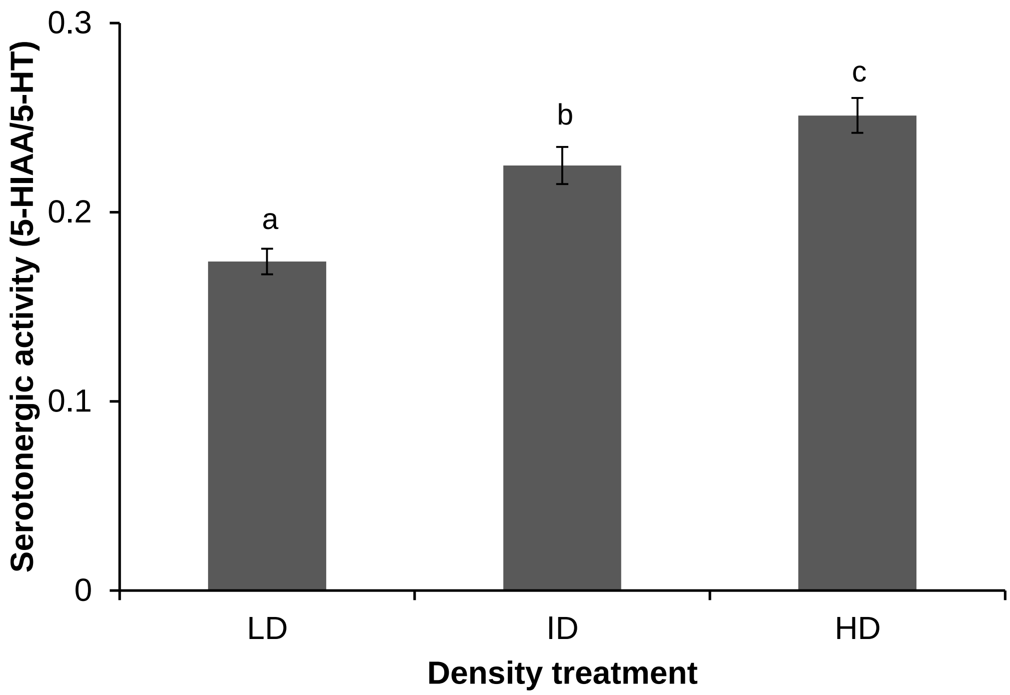
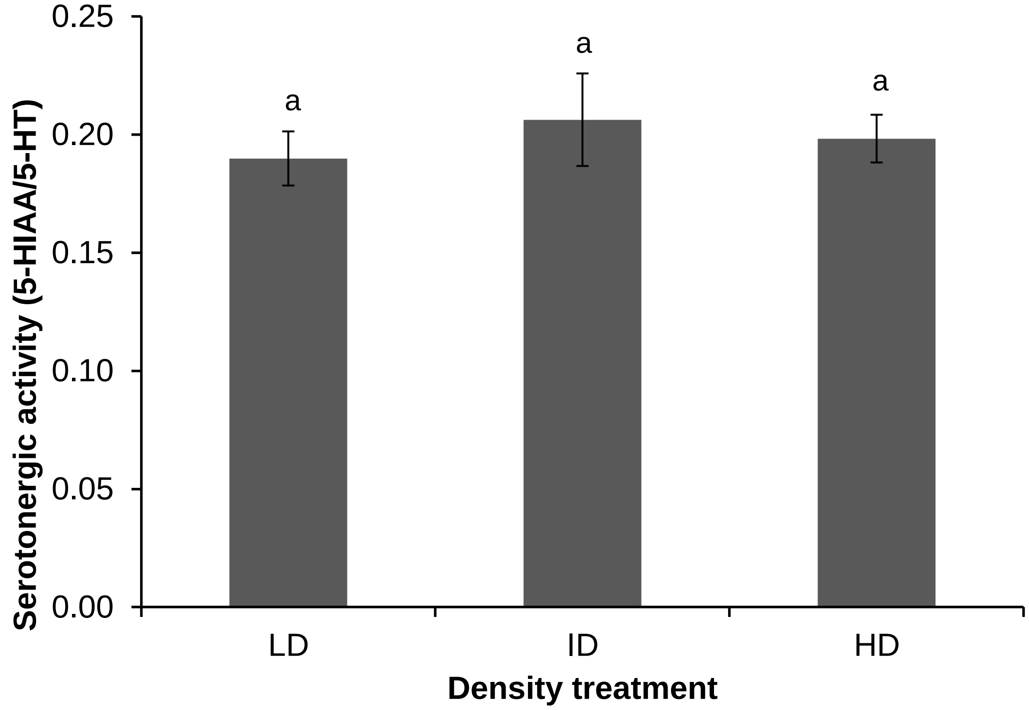


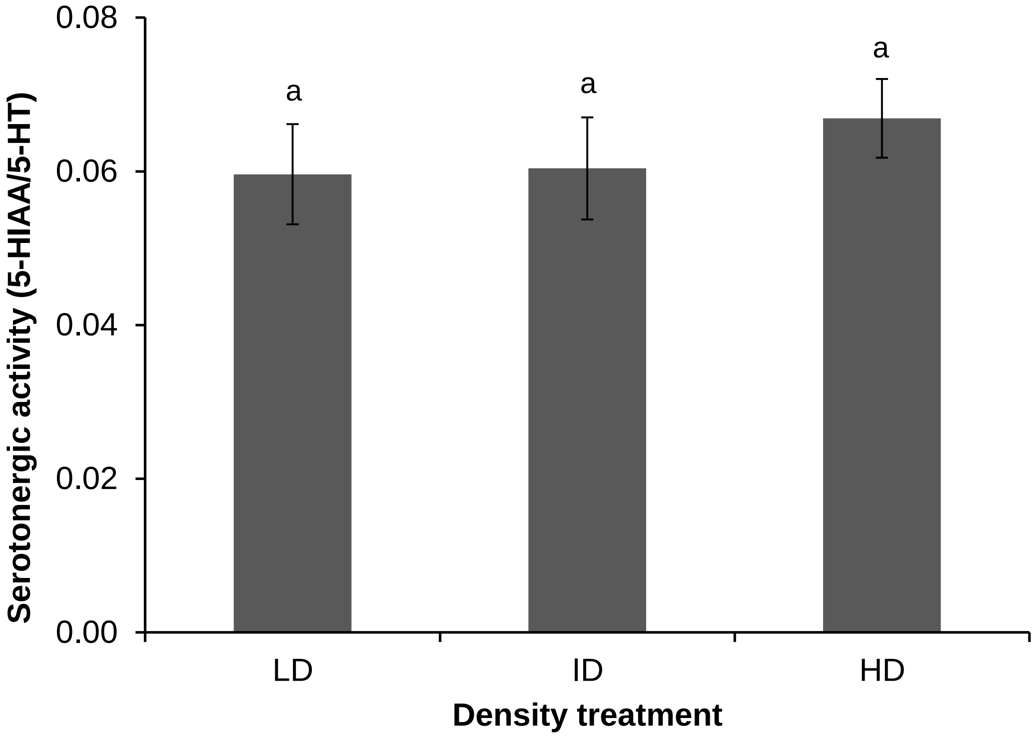
Figure 7. The plasma cortisol concentrations (ng ml^{-1}) of individuals between the density treatments (n=3 tanks per treatment); 25 kg m^{3-1} (LD), 80 kg m^{3-1} (ID) and 140 kg m^{3-1} (HD).



a) Brain stem



b) Telencephalon



c) Hypothalamus

Figure 8. The serotonergic activity level (5-HIAA/5-HT) in the a) Brain stem, b) Telencephalon, and c) Hypothalamus of individuals (n=8 per tank in triplicate) between the density treatments; 25 kg m^{3-1} (LD), 80 kg m^{3-1} (ID) and 140 kg m^{3-1} (HD). The letters denote where the significances lie.

Table 1: The concentration of the metabolite (5-HIAA) and monoamine (5-HT) in the different brain regions of individuals between the different density treatments (n=3 tanks per treatment); 25 kg m^{3-1} (LD), 80 kg m^{3-1} (ID) and 140 kg m^{3-1} (HD).

		Density treatment			
Brain region	Metabolite and monoamine	LD	ID	HD	p value
Brain stem	5-HIAA	51,90 ± 2,32	63,64 ± 3,05	72,07 ± 4,08	0,001
	5-HT	304,35 ± 12,80	296,42 ± 15,04	287,62 ± 11,86	0,632
Telencephalon	5-HIAA	170,69 ± 8,75	171,70 ± 12,08	218,70 ± 16,18	0,006
	5-HT	964,52 ± 67,67	972,80 ± 122,92	1228,45 ± 146, 95	0,106
Hypothalamus	5-HIAA	96,45 ± 5,39	115,38 ± 9,85	113,26 ± 7,72	0,224
	5-HT	1898,0 ± 138,66	2013,82 ± 129,33	1733,67 ± 138,0	0,299

Appendix III

Paper III: Elevated levels energy expenditure results in reduced growth performance in farmed rainbow trout *Oncorhynchus mykiss* reared at low density

Elevated energy expenditure at low rearing density decreases growth performance in rainbow trout *Oncorhynchus mykiss*

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Abstract

In Salmonid fishes, density has been shown to have a profound influence on behaviour, with evidence for an increase display of aggressive behaviour and the formation of dominance hierarchies at low densities. As density increases, it is commonly assumed that aggression decreases, as the cost and effort required to establish and maintain dominance hierarchies increases, leading to an altered behaviour in which schooling becomes more prevalent. The increased energy expenditure associated with aggressive interactions has been identified as one mechanism causing a reduced efficiency in feed utilisation and therefore decreased growth performance. Manipulating aggressive behaviour through density may have advantages from a practical perspective. In the present study the energetic expenditure of rainbow trout held at three densities, 20, 40 and 80 kg m^{-3} were examined, in combination with a conventional growth performance study. In a four week growth experiment, measurements for growth performance and parameters of energetics were investigated at the three densities. Low rearing density caused a significant increase in routine metabolism compared to groups reared at an intermediate and high density, and therefore a higher fraction of the dietary energy intake was used to fuel metabolism. This was linked to a reduced efficiency of feed utilisation and tendency for lower growth performance at low density. This suggests that there may be a bioenergetic advantage of crowding.

Key words: Energetics, aggressive interactions, growth, stocking density, rainbow trout

1. Introduction

Social interactions, in particular aggressive behavioural interactions, between individuals can be energetically costly. This has been demonstrated by increased energetic expenditure associated with agonistic behaviour in, for example, the salmonid fishes sockeye salmon *Oncorhynchus nerka* (Brett, 1964) and rainbow trout *Oncorhynchus mykiss* (Lefrancois *et al.*, 2001; Li and Brocksen, 1977). In territorial salmonid species that form social hierarchies, aggressive behavioural interactions can be intense during the establishment of these hierarchies. It has been found that both the subordinate and dominant status in these hierarchy relationships can carry metabolic costs. Experimental evidence has indicated metabolic disadvantages induced by chronic stress in socially subordinate individuals (Abbott and Dill, 1989; Sloman *et al.*, 2000). On the other hand, territorial defence may also result in greater energy expenditure, which has been reflected in high routine metabolic rates in dominant rainbow trout (Li and Brocksen, 1977).

There is a general lack of information on how density affects the behaviour of fish, presumably due to the difficulties associated with observing fish in large groups. Indirect measures of aggression, such as bite marks or fin damage, have therefore been used to establish the presence of aggressive interactions in groups of fish (Alänärä and Brännäs, 1996; Laursen *et al.*, submitted). Density has been shown to have a profound effect on behaviour in Salmonid species (Bagley *et al.*, 1994). When reared in small groups, rainbow trout can be highly aggressive and form dominance hierarchies (Noakes and Leatherland, 1977; Winberg *et al.*, 1992; Winberg and Lepage, 1998; Øverli *et al.*, 1999). Similarly, there is evidence that dominance hierarchies are formed when rainbow trout are held at low rearing densities of 10 kg m^{-3} (North *et al.*, 2006). Furthermore, bite activity, an indirect measure of aggression, has been observed to be high in rainbow trout at densities of 24 – 36 kg m^{-3} (Alänärä and Brännäs, 1996). As density increases, aggression tends to decrease in salmonids, presumably as the cost and effort required to establish and maintain ordered dominance hierarchies increases, and the frequency of aggressive acts decreases (Alänärä and

Brännäs, 1996; Bagley *et al.*, 1994; Li and Brocksen, 1977). By increasing rearing density, it appears that salmonid fish are forced to switch from damaging aggressive territorial behaviour to schooling behaviour (Grand and Dill, 1999). Indeed, there is evidence that rainbow trout show shoaling behaviour in intensive culture, which has been suggested to reduce aggressive behaviour (reviewed by Ellis *et al.*, 2002).

Although the actual mechanism contributing to growth reduction in fish remains unproven (Ellis *et al.*, 2002), increased energy expenditure has been identified as one mechanism causing a decrease in the efficiency with which fish utilise their feed and their growth performance (Li and Brocksen, 1977). This have been demonstrated in subordinate salmonid fishes where the increased metabolic rate (Sloman *et al.*, 2000) was linked to reduced growth in subordinate individuals (Abbott and Dill, 1989).

If a strong correlation exists between the display of aggressive behaviour and rearing density, this may also explain differences in growth performance at different density levels. For example, in Arctic charr *Salvelinus alpinus*, the effects of social interactions, especially aggressive behaviour, have been attributed to energy requirements and have shown to cause reduced growth at low densities (Brown *et al.*, 1992; Jobling, 1985; Uglem *et al.*, 2009; Wallace *et al.*, 1988). On the other hand, at high densities growth performance was actually improved, which may have been due to a decrease in agonistic interacts and increase in shoaling behaviour at high densities (Brown *et al.*, 1992; Jobling, 1985; Uglem *et al.*, 2009; Wallace *et al.*, 1988). In rainbow trout, elevated energy expenditure resulting in suppressed growth performance at high densities have been attributed to behaviours such as spontaneous swimming activity (Larsen *et al.*, 2012) or social interactions during competition for food (McKenzie *et al.*, 2012).

Therefore, manipulating aggressive behaviour through density may have advantages from a practical perspective. In a parallel study to the current one, the effects of three density levels on indicators of welfare in rainbow trout were explored (Laursen *et al.*, submitted). Here we concluded that individuals in the low density tanks had the space and opportunity to exhibit natural behaviour, in the form of displaced aggression, and that this behaviour was reduced at high density. The aim of the present study was to explore the energetic expenditure of rainbow trout held at the same three densities as in the aforementioned study and the consequences of this on growth performance.

The densities used in these studies are reflective of levels of crowding established in a study by Laursen et al. (2013). In the aforementioned study, the spatial distribution of fish held at different densities in two-tank systems was used to determine density limits accepted by the fish, using indicators of the neuroendocrine stress response to indicate crowding stress. The highest density accepted by the fish showing indications of crowding stress of 140 kg m^{-3} was used here as at the high density, the highest density accepted by the fish without showing indications of crowding stress of 80 kg m^{-3} was used here as an intermediate density and 25 kg m^{-3} was used as a low density. In a four week experiment, measurements for growth performance and parameters of energetics of fish held at these three densities were investigated.

2. Materials and Methods

2.1 Experimental fish

Rainbow trout with an average mass of 130 grams were obtained from a commercial fish farm (Store Restrup, Denmark) for use in the present study. The fish were transported to the Technical University of Denmark, Institute of Aquatic Resources (DTU Aqua) in Hirtshals, where they were quarantined for 15 days upon arrival. Fish in quarantine were fed at 0.75 % of their total body mass per day. Additionally, the salt content in the water was gradually increased to 15 ‰ while the fish were held in quarantine.

2.2 Experimental facilities

Fish were reared in series of 9 circular tanks with a diameter of 1 m and a water level of 0.85 m. Each tank has a central column with a diameter of 0.3 m, giving an average tank volume of 600 litres. Water supply to each tank was derived from a central recirculating system. Water from the system was pumped from the system to the inflow of each tank and left the tank through a central bottom drain. Solids and uneaten feed was collected in a whirl separator fitted to each tank, from which collected solid waste; faeces and uneaten pellets could be collected and quantified. A series of apertures on the vertical inlet pipe generated a high velocity of the incoming water corresponding to a water current of 0.5 body lengths per second (0.5 bl s^{-1}) in each tank. A baseline level of oxygen was supplied to each tank by a small scale oxygen cone diffuser fitted on the inflow of each tank. An oxygen electrode (Oxyguard, Birkerød, Denmark) in each tank measured the oxygen concentrations continuously, and stored all data onto a PLC for later analysis. The experimental tanks were modified to measure oxygen consumption automatically as described by McKenzie *et al.* (2007). Briefly, the water supply to each tank was fitted with a three-way valve; in the first position water supply was derived from the central recirculating system, while in the second position, water in each tank was recirculated. Timing and duration of the positions of the three-way valve was controlled by a digital timer.

2.3 Experimental procedure

Rainbow trout with an average mass of 170 grams stocked randomly into the experimental tanks at densities of 25, 80, and 140 kg m^{-3} in triplicate. Fish were acclimated to the tanks for a period of approximately two weeks during which the feeding level was gradually increased to 1.5% of their biomass per day. At the end of the acclimation period, the number and total mass of the fish in each tank was determined and density was re-adjusted to the initial value.

The experimental period was 28 days, consisting of two 14 day periods. Each period has 12 growth days during which fish were fed, followed by a day of starvation and a day of weighing. Each tank was fed at 1.5 % of the estimated tank biomass per day, with 3 mm pellets (EFICO Enviro 920, BioMar A/S). The fish were fed in the morning at 09:00 using automatic belt feeders that delivered the daily ration over a period of six hours. Following feeding, the solid waste was collected from the whirl separator and the numbers of uneaten pellets were counted to determine feed waste. On the day of weighing, the total biomass in each tank was recorded, and readjusted to the desired density by discarding excess biomass.

During the growth trial, oxygen levels in all tanks were measured and logged continuously at 20 second intervals. The system was programmed so that the three-way valve would recirculate the water in each tank for 8 minutes every hour during the day (09:00 – 17:00) and for 6 minutes during the night (18:00 – 08:00).

Baseline oxygen levels were set at 80% in the tanks held at the low density, 110% at the intermediate density, and 120% at the high density. This was necessary to be able to have identical closing periods despite differences in density, so that the oxygen levels in each tank were similar at the end of the closing period. These differences in oxygen levels were not expected to affect growth, as previous research has shown that growth rate is independent of oxygen concentration above 7 mg L^{-1} (Pedersen, 1987). Water quality parameters; Nitrite (NO_2), Nitrate (NO_3), Ammonia (NH_3/NH_4^+), pH, and temperature (°C) were measured daily to ensure they remained below threshold levels. Threshold levels for $NO_2 < 1$ mg l^{-1}, for $NO_3 < 100$ mg l^{-1}, for $NH_3/NH_4^+ = 0$ mg l^{-1}, and a pH < 8.0. The temperature of the water in the system was thermostatted at 16°C. Light conditions were at 14.5 light and 9.5 dark hours, with the lights automatically switching on at 07:30 switching off at 22:00.

2.4 Measurements

2.4.1. Performance parameters

The specific growth rate (SGR; % bw d^{-1}) was estimated for each tank from the total tank biomass (kg) between two time points using the equation: SGR = 100 * (Ln(W_f) - Ln(W_i)) / time. W_f was the final total biomass in the tank at the end of each growth period, W_i was the total initial biomass in the tank at the start of the growth period, and time (days) was the duration of the growth period. The SGR was estimated for each tank for the first and second growth period.

The feed conversion ratio (FCR; kg kg^{-1}) was calculated for each tank using the equation: (feed intake / biomass gain). Ingested food for the period of investigation was estimated by subtracting the amount of feed waste from the amount of feed given per tank, and biomass gain was Wf – Wi.

2.4.2. Energetic parameters

Calculations of bioenergetics parameters were performed as described in detail by McKenzie et al. (2012) and Larsen et al. (2012). The daily biomass gain per day was calculated for each tank using the equation: W_i + ((W_i / 100) * SGR). For calculating the mass gain on each consecutive day of the growth period, the biomass from the previous day was used as W_i. Based on the daily biomass gain, a period of days was chosen for each tank when the individual growth was similar between all of the tanks. In the present study, the days from when the fish grew from 190 to 220 grams was chosen. Total feed intake, total oxygen consumption for this time period, the energy content of the feed given (see section 2.3) and an oxycalorific coefficient were used to calculate the energetic parameters for each tank. The total feed intake was estimated as the sum of the daily feed intake for the selected time period. The total oxygen consumption was determined as the sum of the daily oxygen consumption rates for the selected time period. The daily oxygen consumption rates were calculated from the slope value obtained from the decline in oxygen in the tank, the estimated total biomass of fish in the tank on the day (using the SGR) and the total volume of water in the tank on the day. The gross energy content of 21.5 (Kj g^{-1}) in the feed was determined from the feed manufacturer information (Biomar A/S). An oxycalorific coefficient of 14.06 Kj g^{-1} O_2 (Dejours, 1981) was used.

2.5 Statistical analysis

A one-way analysis of variance (ANOVA) was used to analyse the energetic parameters, with density treatment (25, 80 and 140 kg m^{-3}) as the independent variable. Correlations between were analysed using a Spearman R test.

The specific growth rate (SGR) and feed conversion ratio (FCR) were analysed using two-way analysis of variance (ANOVA), where density treatment (25, 80 and 140 kg m^{-3}) and growth period (1 & 2) were the independent variables and the dependent variable was SGR or FCR respectively. If a significant difference for an independent variable was found, a Tukey's post hoc test was carried out to determine where the significances were present.

All statistical analyses were carried out using the computer program Statistica (StatSoft, Inc. USA, version 11). The values presented are mean ± standard error.

3. Results

3.1. Energetic parameters

The energetic parameters are summarised in Table 1. The values for each parameter are presented as means ± s.e. (N=3 per density treatment). There was a significant effect of rearing density in the amount of total energy that was dissipated for metabolism ($p = 0.005$). Specifically, the total energy used for metabolism was higher at the low density compared to the intermediate density ($p \leq 0.05$) and high density ($p \leq 0.001$), as a result of the higher total oxygen used at the low density compared to the intermediate ($p \leq 0.05$) and high density ($p \leq 0.05$). The total energy allocated for growth was significantly affected by density ($p = 0.034$), with a higher value at the low density compared to the intermediate ($p \leq 0.067$) and high density ($p = 0.971$). This was due to the significantly higher total energy intake ($p \leq 0.05$) at the low density compared to the intermediate ($p \leq 0.05$) and high density ($p \leq 0.05$), as a consequence of a higher total feed intake at the low density ($p \leq 0.05$) than the intermediate ($p \leq 0.05$) and high density ($p \leq 0.05$) during the selected period when the fish grew between 190 and 220 grams. Although the daily feed intake was similar for each density, the number of days to grow during the selected period was higher at the low density compared to the intermediate and high density, resulting in a higher total feed intake. The cost of growth ($p \leq 0.05$) was higher for the individuals held at the low density than at the intermediate ($p \leq 0.05$) and high density ($p \leq 0.05$). Furthermore, the negative relationship between SGR and the total energy consumed for metabolism (R^2=0.874, $p \leq 0.001$; Fig. 1).

3.2. Performance parameters

The growth performance parameters are summarised in Table 2. The values for density start and density end, SGR and FCR are represented as means ± s.e. of the two growth periods (N=6 per density). There was a significant effect of density ($p \leq 0.040$) on the specific growth rate pooled for both growth periods, with a tendency for a lower SGR at the low density compared to the intermediate ($p \leq 0.073$) and high density ($p \leq 0.057$). There was no effect of growth period ($p = 0.163$) or interaction between growth period and density ($p < 0.228$) on the specific growth rate (SGR). There was a significant effect of density ($p \leq 0.019$) on the feed conversion ratio pooled for both growth periods. The FCR was higher in the low density compared to the intermediate density ($p = 0.045$) and the high density ($p = 0.026$). There was no effect of growth period ($p \leq 0.1$) or interaction between density and growth period ($p \leq 0.137$) on the FCR.

4. Discussion

The results in the present study demonstrate an increased energy expenditure in individuals held at 25 kg m^{-3}, as evidenced by a higher total energy dissipated for metabolism at low density compared to the intermediate and high densities. In a parallel study (Laursen *et al.*, submitted) we investigated the effects of the same density levels on welfare. Here we demonstrated neuroendocrine evidences (elevated central signalling of serotonin and low plasma levels of cortisol) of chronic stress in high densities, while no such indications were shown in low densities. However, scale loss indicated higher levels of aggression in low densities, and it was suggested that stress reducing displaced aggression was constrained in high densities which led to chronic stress. Due to the practical difficulty of carrying out quantitative behavioural observations of individual fish at such high stocking densities, the indirect measures of aggression obtained in the parallel study were used as an indication of increased aggressive behavioural interactions at the different densities. Here, it is therefore speculated that the individuals in the low density tanks were wasting energy through displaying aggressive social interactions. Indeed, social interactions have been demonstrated to be energetically costly. In small experimental populations, salmonid fishes can be highly aggressive and form dominance hierarchies. Both dominant and subordinate individuals have been found to have higher metabolic rates (Abbott and Dill, 1989; Li and Brocksen, 1977; Sloman *et al.*, 2000).

The individuals held in the tanks at the high density had the lowest energy expenditure in this study, as indicated by the lower energy dissipated for metabolism at this density. Using the same line of reasoning as above, this suggests that aggressive behaviour was low in the high density tanks. Indeed, in the parallel welfare study, scale loss was found to be low at this density (Laursen *et al.*, submitted). Furthermore, in other Salmonid species, such as Arctic charr *Salvelinus alpinus*, a decrease in agonistic interactions and an increase in shoaling behaviour at high densities were attributed to result in lower energy expenditure (Brown *et al.*, 1992; Jobling, 1985; Uglem *et al.*, 2009; Wallace *et al.*, 1988).

There were implications of energy expenditure on the growth performance at the different densities. As a consequence of the high energy expenditure at low density, there was a less efficient utilisation of feed resulting in a tendency for a reduced growth performance at low density. On the other hand, the lower energy expenditure at high density resulted in a tendency for a better growth performance. A stimulatory effect on growth has even been found at high rearing densities in some species of salmonid fishes. For example, in Arctic charr, *Salvelinus alpinus*, growth performance was suppressed at low density and was actually improved at high density, for a density of up to at least 100 – 150 kg m^{-3} (Brown *et al.*, 1992; Jobling and Baardvik, 1994; Jørgensen *et al.*, 1993; Wallace *et al.*, 1988). Taken together, the results suggest that there may be bioenergetic advantages to crowding from a production point of view.

It is appreciated that the results presented here for growth performance differ in comparison to other studies on the effects of rearing density in rainbow trout. Previous research has established a general consensus that increasing stocking density adversely affects growth performance (reviewed by Ellis *et al.*, 2002). However, in light of more recent studies, it becomes evident that a multitude of confounding factors are involved, illustrating the complex nature of how rearing density affects growth performance. For example, (Boujard *et al.* (2002) investigated the effect of different densities combined with different levels of food accessibility, and found that reduced food intake and growth at high density was due to food accessibility and not crowding stress. In another example, the combined effects of stocking density and water quality were studied by Person-Le Ruyet et al. (2008). These results showed that growth performance was best under high water quality conditions for all densities, although that growth performance was worst at the highest density despite not observing any major physiological disturbances (Person-Le Ruyet *et al.*, 2008). Furthermore, the complexity of rearing density is further illustrated by the fact that there are several studies showing no effects of density on growth performance in rainbow trout (Bagley *et al.*, 1994; Kebus *et al.*, 1992; North *et al.*, 2006), which contradicts the general perception that stocking density *per se* suppresses growth performance in rainbow trout (Bagley *et al.*, 1994).

Although water current was neither a focus nor a variable in the present study, it cannot be excluded that it may have had an effect on the results. The motivation for providing a slow water current of 0.5 bl s^{-1} was to promote schooling behaviour in the fish in an effort to minimise impulsive swimming activity and aggression, often associated with being held in still water, which may present substantial energetic costs in Salmonids (Davison, 1997). In this manner, fish would swim in a unidirectional fashion against the current and during feeding times the pellets would be more evenly distributed around the tank. Furthermore, a moderate water current speed was provided to avoid energetic costs associated with high swimming speeds. A swimming speed of 0.5 bl s^{-1} was chosen as an intermediate between swimming at 0.2 bl s^{-1}, which is considered resting velocity (Woodward and Smith, 1985), and the optimal swimming speed, defined as the lowest cost of transport, which is between 1.1 and 1.2 bl s^{-1}, depending on rearing history (Skov *et al.*, 2011). However, presumably this water current velocity was not sufficient to fully eradicate undesirable behaviour at low rearing density.

5. Conclusion

The results presented here showed that energy expenditure was elevated at the low density, compared to the intermediate and high density, as demonstrated by a higher energy dissipated for metabolism. It was speculated that is may be due to higher aggression in the tanks at the low density. Energy expenditure was linked to a reduced efficiency of feed utilisation and tendency for lower growth performance and slower at low density. In contrast, this resulted in a tendency for a better growth performance at the intermediate and high density, which suggests that crowding confers a bioenergetic advantage for rainbow trout.

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Table 1. Energetic parameters at each density (25, 80 & 140 kg m $^{-3}$) for the selected time period when the fish in each tank had a similar mass gain, from 190 to 220 grams. N=3 tanks per density

	Density (kg m $^{-3}$)		
Parameter	25	80	140
Estimated mass gain (g fish $^{-1}$)	29.71 ± 0.15	30.14 ± 0.51	31.33 ± 0.34
Specific growth rate (% bw d $^{-1}$)	1.16 ± 0.03	1.49 ± 0.09	1.62 ± 0.19
Time period of mass gain (days)	12.67 ± 0.33	10.00 ± 0.58	9.67 ± 1.20
Mean body weight (g)	203.4 ± 0.79	203.49 ± 0.18	203.08 ± 0.81
Daily feed intake (g kg d $^{-1}$)	15.34 ± 0.05	15.09 ± 0.05	14.72 ± 0.06
Total feed intake (g kg $^{-1}$)	195.17 ± 5.89	150.77 ± 8.92**	138.63 ± 12.86**
Total O_2 used (mg kg $^{-1}$)	97479.38 ± 3162.03	70023.66 ± 3615.41**	57073.34 ± 8241.47**
Total Energy intake (Kj kg $^{-1}$)	4196.21 ± 126.68	3241.65 ± 331.87**	2980.44 ± 510.01**
Total Energy dissipated for metabolism (Kj kg $^{-1}$)	1325.11 ± 42.98	951.88 ± 69.33**	775.84 ± 231.88***
Total Energy dissipated for metabolism (%)	31.60 ± 0.95	29.42 ± 0.78	25.79 ± 2.73
Total Energy allocated for growth (Kj kg $^{-1}$)	2871.10 ± 110.36	2289.76 ± 262.54*	2204.60 ± 280.48*
Total Energy retained for growth (%)	68.40 ± 0.95	70.58 ± 0.78	74.21 ± 2.73
Cost of growth (Kj g $^{-1}$)	19.67 ± 0.88	15.45 ± 1.63**	14.29 ± 2.18**

A triple asterix (***) indicates a significant difference of $p < 0.01$, a double asterix (**) indicates a significant difference of $p < 0.05$ and a single asterix (*) indicates near significant levels ($p < 0.10$), compared to low densities.

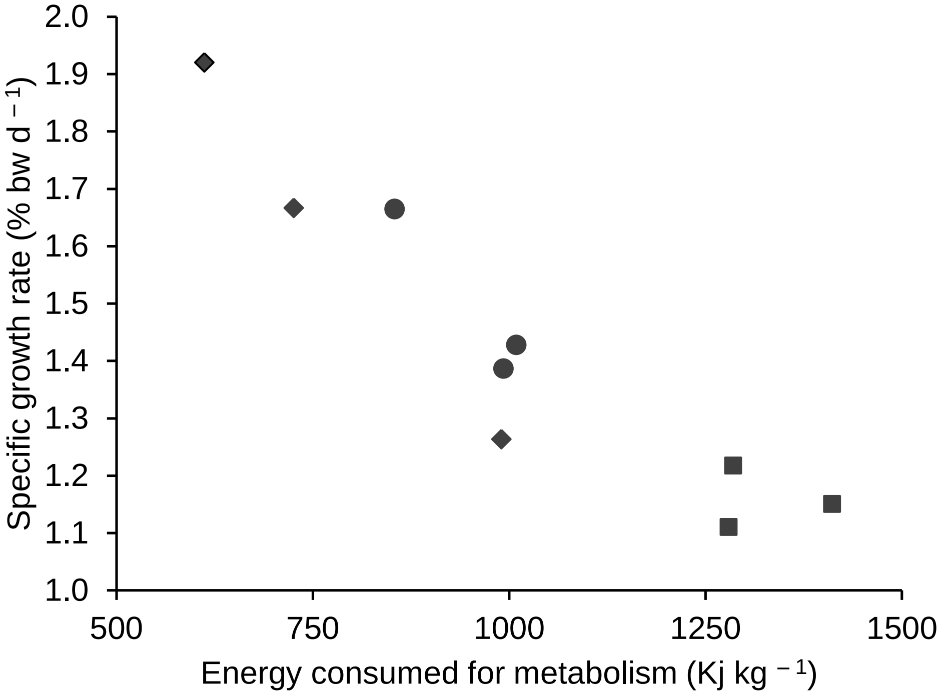

Figure 1. The relationship between the specific growth rate (% bw d^{-1}) and the total energy consumed for metabolism (Kj kg^{-1}) for the selected time period when the fish in each tank had a similar mass gain, from 190 to 220 grams. Triangles denote the 140 kg m^{-3} (High) tanks, circles denote the 80 kg m^{-3} (Intermediate) tanks and squares denote the 25 kg m^{-3} (Low) tanks (n=3 tanks per density).

Table 2. Performance parameters at the rearing densities of 25, 80 & 140 kg m $^{-3}$ represented as means from both growth periods. N=6 tanks per density

	Density (kg m^{-3})		
Parameter	25	80	140
Density start (kg m $^{-3}$)	25,19 ± 0,13	80,18 ± 0,10	140,22 ± 0,05
Density end (kg m $^{-3}$)	29,12 ± 0,17	94,82 ± 0,41	166,06 ± 1,45
Survival (%)	99,81 ± 0,19	99,62 ± 0,03	99,38 ± 0,13
Specific Growth Rate (% bw d $^{-1}$)	1,12 ± 0,04	1,29 ± 0,03*	1,30 ± 0,07*
Feed Conversion Ratio (kg kg $^{-1}$)	1,28 ± 0,05	1,08 ± 0,03**	1,05 ± 0,05**

A double asterix (**) indicates a significant difference of $p < 0.05$ and a single asterix (*) indicates near significant levels ($p < 0.10$), compared to low densities.

DTU Aqua – National Institute of Aquatic Resources – is an institute at the Technical University of Denmark. DTU Aqua's mission is to conduct research, provide advice, educate at university level and contribute to innovation in sustainable exploitation and management of aquatic resources. We investigate the biology and population ecology of aquatic organism, aquatic physics and chemical pro-cesses, ecosystem structure and dynamics, taking account of all relevant natural and anthropogenic drivers.

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