INVESTIGATIONS INTO THE FORMATION OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS)* SOCIAL HIERARCHIES AND POSSIBLE HIERARCHICAL DISRUPTION BY AN ENVIRONMENTAL PERTURBATION

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Josias (Si) Grobler, B.A. Biology

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AUTHOR: Josias (Si) Grobler, B.A. Biology (Dordt College, Sioux Center, Iowa)

SUPERVISOR: Dr. Chris M. Wood

COMMITTEE MEMBERS: Dr. Sigal Balshine, Dr. James Quinn

UNOFFICIAL COMMITTEE MEMBER: Dr. Katherine Sloman

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Abstract

Salmonids, such as rainbow trout, form social hierarchies, consisting of dominant and subordinate individuals, when in groups in environments with limiting resources, such as space or food. There were two main objectives for this thesis: to investigate the physiological consequences of being in a social hierarchy as well as to investigate whether grouped social status or individual behaviour most accurately recorded physiological data for a hierarchy and secondly, to investigate the behavioural and physiological changes of groups of rainbow trout exposed to ammonia concentrations which are above chronic protected guidelines.

To create social hierarchies, groups of four fish were fed by a new method using a darkened feeding container, twice daily (morning and evening) for eight days. Each morning feeding was videotaped in order to record aggressive behaviour. Each aggressive act was scored, allowing for fish to be assigned a social status. For ammonia exposures, groups of fish were exposed to either 700, 1200 and 1500 µM total ammonia (or 2.97, 5.10, 6.37 µM NH_3, respectively) 24 hours before first feeding and these concentrations were maintained throughout the experiment. On day 5 and day 10, physiological parameters were taken in fish fasted for 24-h in control and 700 µM total ammonia exposed hierarchies.

Social hierarchies were created in all ammonia-free and 700 µM total ammonia groups, with no hierarchies formed in 1200 and 1500 µM total ammonia groups. In

ammonia-free hierarchies, one fish would become dominant, while the three subordinate individuals would each assume a stable social rank and display similar physiology which was different from the dominant fish.

Fish from the 700 μM total ammonia group showed reductions in various physiological parameters during period 1, however, these fish displayed similar values as what was reported in ammonia-free hierarchies during period 2. This suggests biochemical or physiological changes occurring in these fish in order to acclimate to the high ammonia environment.

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Thesis format and organization

This thesis is presented in a 'sandwich thesis' format, consisting of four chapters. Chapter one consists of a general introduction with relevant background. Chapters two and three are experimental chapters which are formatted for publication in peer-reviewed journals. Finally, Chapter four summarizes major findings and implications along with a section dedicated to possible future research.

Chapter 1: **General introduction**

Chapter 2: **Rainbow trout hierarchies created under laboratory conditions: two approaches to investigate hierarchical structure and individual physiology**

Authors: Josias M.B. Grobler and Chris M. Wood

Chapter 3: **High external ammonia's effect on the formation of rainbow trout hierarchies and resulting physiological changes**

Authors: Josias M.B. Grobler and Chris M. Wood

Chapter 4: **General conclusion**

Table of contents

List of figures

Chapter 2

Chapter 3

Chapter 1: General introduction

Social hierarchies

Groups of organisms will form dominance hierarchies in environments with limiting resources, such as space, food or mates (Chapman, 1966). These hierarchies are necessary in order to avoid excessive fighting over limiting resources and reduce individual energetic costs (Gurney, 1979). As such, a linear 'pecking order' is established through agonistic behaviour, such as fighting, protection and intimidation within these groups. Drews (1993) provides a good working definition of dominance: "the pattern of repeated, agonistic interactions between two individuals, characterised by a consistent outcome in favour of the same dyad member and a default yielding response of its opponent rather than escalation". Depending on the species and the limiting resource, a wide combination of aggressive behaviours might be observed.

Salmonids can form social hierarchies under natural (Keenleyside and Yamamoto, 1962), semi-natural (Sloman et al., 2008), and laboratory (Adams et al., 1998) settings. However, much of our knowledge of fish hierarchies has been gathered from laboratory settings which most likely differ from the natural environment in size, complexity as well as time scale. This makes relating any laboratory findings to what might occur in the wild extremely difficult. But interesting insights into how dominance hierarchies might function in the environment have been attained through intensive laboratory studies.

For example, it is well established that social hierarchies consist of dominant and subordinate individuals, with dominant fish out-competing other fish to have preferential access to the limiting resource (McCarthy et al., 1992; Adams et al., 1998). Dominant individuals usually have greater growth rates compared to subordinates (Pottinger and Pickering, 1992; Sloman et al., 2000a). This lowered growth in subordinates can be partially attributed to reduction in food consumption resulting from direct competition with the dominant fish. However, Abbot and Dill (1989) showed that equal food rations between dominant and subordinate individuals still resulted in reduced growth in the subordinate fish, suggesting that other factors are at play regarding reduced growth. Indeed, Sloman et al. (2000c) demonstrated that subordinate fish have a higher metabolic demand due to stress which would also contribute to lowered growth. Other physiological profiles that subordinates tend to have include greater physical damage and lowered immunity (Peters et al., 1988; McCarthy et al., 1992). Taken together, this would suggest that dominant individuals are favoured compared to subordinates, however, dominant fish might have greater predation risk due to increased aggressive behaviours (Jakobsson et al., 1995).

Another physiological consequence that is often reported in subordinate individuals is elevated plasma cortisol levels (Pottinger and Pickering, 1992; Sloman et al., 2000b). Cortisol is produced in teleost fish in order to 'cope' with a stressor, such as a dominance hierarchy (Wendelaar Bonga, 1997). High concentrations of cortisol have

been shown to increase oxygen consumption, increase mobilization of energy stores (Morgan and Iwama, 1996; De Boeck et al., 2001) and reduce immunity (Wendelaar Bonga, 1997). There is also evidence to suggest that elevated plasma cortisol might be a predictor of social status, as high plasma cortisol levels caused rainbow trout (*Oncorhynchus mykiss)* to become submissive (Sloman et al., 2001; Gregory and Wood, 1999).

Between pairs and among small groups of fish, the cortisol response appears to be more variable in the latter, with some studies reporting elevated plasma cortisol levels in subordinates (Ejike and Schreck, 1980; Winberg and Lepage, 1998), while others reporting no difference between dominant and subordinate individuals (Pottinger and Pickering, 1992; Sloman et al., 2000a). This difference could be due to the environment in which the hierarchy was established as well as the experimental methodology used in order to obtain cortisol measurements. Indeed, in a recent study, Sloman et al. (2008) reported that in a complex, natural stream, dominant fish exhibited higher plasma cortisol. This suggests that the physiology observed in fish of laboratory-created social hierarchies might be different than in hierarchies occurring in a less controlled environment (aquaculture or natural setting).

Ammonia in the aquatic environment

Ammonia in the aquatic environment exists in two forms, NH_3 and NH_4^+ (pK= ~9.5). NH_3 is a non-polar gas which can be protonated to form NH_4^+. As a non-polar gas, NH_3 is generally considered the more toxic form towards aquatic organisms because it can easily diffuse across cell membranes. The speciation between NH_3 and NH_4^+ is dependent on various environmental factors, such as, temperature, salinity, atmospheric pressure, and pH, with the latter being the most important (USEPA, 1999).

Ammonia is a unique toxicant in fish given that ammonia is naturally produced from the catabolism of protein internally. Fish have to constantly excrete ammonia as part of a large detoxification mechanism. This is accomplished through passive diffusion down ammonia's concentration gradient via the gills. There is now strong evidence to suggest that there are specific ammonia transport proteins, Rh channels, in the gills that facilitate ammonia diffusion (Wright and Wood, 2009), so branchial excretion may occur by both simple passive diffusion and facilitated diffusion. However, Rh channels are bidirectional transporters (Nawata et al., 2010), so during elevated ammonia conditions, this concentration gradient might be reversed resulting in decreased levels of ammonia excretion or even net ammonia loading, both of which lead to elevated plasma ammonia concentrations in fish (Wright and Wood, 2009). Convulsions and death are the end result if internal ammonia burden reaches toxic thresholds (Randall and Tsui, 2002).

Ammonia toxicity is likely the result of glutamate build-up within neurons. It has been proposed that high levels of ammonia in the brain result in high levels of glutamate

by either increasing glutamate release and/or decreasing glutamate synaptic reuptake (Rao et al., 1992; Randall and Tsui, 2002). Following this, NMDA type glutamate receptors are activated which causes an influx of Ca^{2+} into the cell which ultimately results in neuronal cell death. However, NMDA might be activated before increases in glutamate occur by NH_4^+ substituting for K^+ and leading to depolarization of the neuron (Hermenegildo et al., 2000). ATP depletion can occur due to NMDA activation, causing the sodium-dependent glutamate mechanism to work less effectively, resulting in an increase of intercellular glutamate.

Another way to reduce internal ammonia burden, besides ammonia excretion, is to convert ammonia into less toxic substances. For example, depending on the species of fish, ammonia can be converted to urea or glutamine. Urea is produced via the ornithine urea cycle during air exposures (documented in Singhi catfish - Saha et al., 2001) or during high environmental pH (seen in Lake Magadi tilapia - Randall et al., 1989). Urea can then be expelled into the environment through the gills or via the urine. In rainbow trout, ammonia levels are reduced through the conversion of ammonia to glutamine by glutamine synthetase and glutamate dehydrogenase (Randall and Tsui, 2002; Wright et al., 2007). Starting from α-ketoglutarate, NH_4^+ is added via glutamate dehydrogenase to produce glutamate which is then used to make glutamine by adding another NH_4^+ through glutamate synthetase. Two moles of NH_4^+ will be detoxified for every glutamine produced. Glutamine can be easily stored in the tissues until normal conditions return

where upon glutamine can be utilized as an oxidative substrate. However, this process is energy demanded, requiring two moles of ATP for every mole of NH_4^+ removed (Randall and Tsui, 2002).

Before convulsions or death occurs, many non-lethal effects of high ammonia may be seen in fish. One of the most commonly cited side effect is a reduction in food consumption (Beamish and Tandler, 1990; Wicks and Randall, 2002; Ortega et al., 2005). Appetite reduction is mediated, at least in part, by serotonin (5-HT) which has been shown to increase in the brain in a dose-dependent fashion with increasing concentrations of external ammonia (Ortega et al., 2005).

In dominance hierarchies, where unequal feeding occurs, differential serotonin concentrations between dominant and subordinate individuals have also been observed, with dominant fish displaying lower serotonergic activity (Winberg et al., 1993). This study provides compelling evidence in relating increased serotonergic activity with reduced food intake and the establishment of hierarchical structure. Increased plasma cortisol is also reported to occur in elevated ammonia conditions (Person-Le-Ruyet et al., 1998; Ortega et al., 2005), and cortisol itself can also reduce feeding (Gregory and Wood, 1999). These two factors are intimately involved in dictating the behaviour of fish in a hierarchy.

Since high environmental ammonia and dominance hierarchies can influence both food consumption and plasma cortisol in fish, investigating the simultaneous effect of both factors on fish behaviour and physiology provides a unique situation, in some ways similar to that which may occur in an aquaculture setting.

Dominance hierarchies in aquaculture

Knowledge of social hierarchies in salmonids is particularly important to the aquaculture industry. In either land-based or water-based fish farming, space and food are restricted. These conditions can lead to hierarchies forming, resulting in unequal growth and diminished health for some of the fish. To prevent dominance hierarchies from forming, several strategies have been implemented to reduce the competitiveness in aquaculture settings. These include, but are not limited to, manipulations of: density of fish, food quantity, food quality, water flow and water quality (see Ashley, 2007 for an exhaustive review).

Density refers to the weight of the fish per unit volume (Ellis 2001). 'Overcrowding' (having more individuals inhabiting an environment than the carrying capacity allows) most often leads to high stress and poor water quality, resulting in poor health for the fish. Overcrowding, however, may also reduce aggression within the group (Fenderson and Carpenter, 1971) which could result in a less established hierarchy being formed. As well, dispensing enough food at irregular times will also reduce the

competitiveness within a group of fish. Changing the nutrition of the feed can also influence fish behaviour. A high level of dietary L- tryptophan has shown to suppress aggression (Winberg et al., 2001). The surrounding environment could also be modified to cause a reduction in aggression. Sloman et al. (2002) have shown that hierarchies become less stable during increased water flow. However, in such a circumstance, growth might be jeopardized. It is clear that there are several techniques available that can create an environment in which it is difficult for fish to monopolize a limited resource and thus reduce the chance of a hierarchy forming.

Lastly, water quality plays a substantial role in determining the health of a population of fish. It has been assumed that near pristine water conditions are needed for the highest health and highest growth to occur in an aquaculture setting. Pristine environments, however, might be expensive as well as difficult to maintain. For example, a spike in total ammonia concentrations after a meal has been shown to be detrimental towards the health of fish (Foss et al., 2009). Conversely, Wood (2004) and Madison et al. (2009) exposed rainbow trout and walleye, respectively, to moderate concentrations of total ammonia (70 and 225, 100 and 300 µM total ammonia, respectively) and demonstrated an increase in growth compared to control fish. This was due to improved protein synthesis caused by either 'backing-up' endogenous ammonia so that it was incorporated in protein synthesis, and/or due to decreased energy expenditure associated with the ammonia exposure.

Main objectives

With knowledge that salmonids do form dominance hierarchies in laboratory settings and that environmental factors affect their behaviour, two separate investigations were conducted.

The first main objective was to establish the physiological profile that might exist within a dominance hierarchy consisting of four individual rainbow trout. This study attempted to investigate how social interaction might influence the physiology of fish exposed to a hierarchy over many days. Behavioural parameters were correlated with physiology using two statistical methods: social rank based on position within a specific hierarchy or each individual's unique behaviour. This was done in order to discover which method leads to a greater understanding of both physiology and behaviour. A new non-invasive method was developed to create the necessary aggression to cause a hierarchy. Manipulation of this method allowed physiological parameters to be recorded.

I hypothesized that a social hierarchy would result, consisting of unique physiological characteristics for each social status. For example, the most dominant fish would display physiology that was different than all the other fish and the least dominant individual would have a unique physiological profile compared to the rest of the fish.

The second objective of this thesis was to investigate how robust this social hierarchy is when faced with an extreme environmental perturbation, such as elevated waterborne ammonia. Ammonia was chosen due to its emerging relevance in the aquatic environment. This is a reflection of the globally increasing problem of nitrogen mobilization and resulting ammonification of natural waters through the overuse of fertilizers (Vitousek et al., 1997), as well as a substantial body of literature documenting the many effects that ammonia has on fish physiology (Tsui and Randall, 2002). In particular, ammonia has been shown to influence growth, swimming performance, and cortisol response, all of which are important factors that influence the social position of fish inside a dominance hierarchy. Rangefinder tests were performed to identify concentrations of ammonia that do and do not allow for dominance hierarchies to be established. I hypothesized that high yet still sublethal concentrations of ammonia would completely prevent a hierarchy from forming. Physiological parameters were recorded at an ammonia concentration where dominance hierarchies still formed so as to investigate the specific effects that elevated ammonia burden has on the structure of the hierarchy and the physiology of individuals within the hierarchy. Specifically, I hypothesized that the high external ammonia would reduce appetite and swimming performance in dominant fish so that subordinate individuals would be healthier than in control hierarchies. The level of ammonia chosen for these tests (700 μM total ammonia or 2.97 μM NH_3 at pH 7.2) proved to be somewhat higher than allowable water quality guideline values for chronic ammonia exposures. Nevertheless, the interaction between behaviour

and ammonia toxicity reflects a realistic scenario of what could happen in aquaculture or in a wild setting.

Lastly, the possible stress associated with the non-invasive methodology used to record physiological parameters was assessed by measurements of plasma cortisol.

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Chapter 2: Rainbow trout hierarchies created under laboratory conditions: two approaches to investigate hierarchical structure and individual physiology

Abstract

Salmonids, such as rainbow trout, form social hierarchies when in groups in environments with limiting resources, such as space or food. The objective of the study on juvenile rainbow trout was to investigate the physiological consequences of being in a dominance hierarchy as well as to investigate whether grouped social status or individual behaviour most accurately recorded physiological data for a hierarchy.

To create a social hierarchy, groups of four fish were fed using a darkened feeding container, twice daily (morning and evening) for eight days. Each morning feeding was videotaped in order to record aggressive behaviour. Each aggressive act was scored, allowing for fish to be assigned a social status. On day 5 and day 10, physiological parameters were taken in fish fasted for 24-h.

Social hierarchies were created in all tested groups of four rainbow trout. One fish would become dominant, while the three subordinate individuals would each assume a stable social rank. When classified according to this social rank, the three subordinate individuals all displayed similar physiology, different from the more favourable physiology in the dominant fish. The latter included greater feeding, higher specific growth rate, greater increase in condition factor, lower oxygen consumption, higher ammonia excretion, and greater protein utilization in aerobic metabolism.

However, when individual aggression was taken into account, a gradient was observed between aggression and physiology. As aggression increased, various other physiological parameters changed in parallel, regardless of social status. This suggests that individual behaviour needs to be considered instead of just social status when studying hierarchies in rainbow trout.

1. Introduction

Salmonids will form social hierarchies in settings with limiting resources such as food and space (Chapman, 1966). Establishment of this 'pecking order' is accomplished through agonistic behaviour, where fish within a group use aggression to try and out-compete other fish for the limiting resource. Based on the aggression displayed, fish can be classified as either dominant (more aggressive) or subordinate (less aggressive) individuals (Glimour et al., 2005).

Stable hierarchies (in which social status does not change) are beneficial to both subordinate and dominant individuals by reducing aggressive behaviour compared to unstable hierarchies (Gurney and Nisbet, 1979). However, dominant individuals are often viewed as the 'winners' in the hierarchy, displaying higher growth rates, higher food consumption and having access to mates (Pottinger and Pickering, 1992; McCarthy et al., 1992). Subordinates, on the other hand, are seen as the 'losers', exhibiting physical damage, lowered immunity, and slower growth rates (Abbot and Dill, 1989; Peters et al., 1988; McCarthy et al., 1992).

Many studies have also shown that subordinate fish have elevated plasma cortisol levels (Pottinger and Pickering, 1992; Sloman et al., 2000b; Hoglund et al., 2002) resulting from social hierarchies. The magnitude of the cortisol rise has been correlated with the strength of a hierarchy (Sloman et al., 2001). However, these studies were conducted using pairs of salmonids and are probably an oversimplification of what might occur within groups fish, for example, in an aquacultural or natural setting.

Other physiological parameters have also been correlated to lower social status. Higher metabolic rate has been documented to occur as a result of subordination (Sloman et al. 2000c). Unequal feeding, which can occur in a dominance hierarchy, can lead to differential ammonia excretion rates between fish (Alsop and Wood, 1997; Bucking and Wood, 2008). However, these parameters have not been recorded within a social hierarchy.

There are several factors that determine whether or not an individual will attain a dominant social position. A few of these are: basal cortisol levels (Gregory and Wood, 1999), prior social experience (Cutts et al., 1999) and feeding motivation (Johnsson et al., 1996). However, metabolic rate might be the most important factor for determining dominance. Fish with high resting metabolism tend to achieve high social status (Metcalfe et al., 1995). These fish have higher energetic demands resulting in higher aggression.

It has become common place while investigating social status in fish hierarchies to group together fish of equal social status from different hierarchies. However, depending on the study, authors have correlated individual aggression with individual physiology. Lahti et al. (2001) reported aggressiveness per population of brown trout and specific growth rate of the population, and noticed a tendency for trout populations that perform high aggression to have high growth rates. Sloman et al. (2008) also correlated higher

individual behavioural scores to higher plasma cortisol levels and higher growth rates. But studies recording both group and individual data in hierarchies are limited.

The objectives of this study were two-fold. The first was to use non-invasive methods (visual observations of behaviour, respirometry of individuals, growth and feeding measurements) to investigate how social status affects the physiology of individual trout in a dominance hierarchy consisting of more than two animals. We were particularly interested in ammonia excretion rates, which when measured together with oxygen consumption rates, provide an indication of the degree to which proteins and amino acids are used to fuel aerobic metabolism (van den Thillart and Kesbeke, 1978). I hypothesized that each social status would display a distinct and unique physiological profile from each other. The second objective was to examine whether physiological data for a hierarchy are more accurately analysed on the basis of grouped social status or on the basis of individual behaviour.

2. Methods and Materials

2.1. Experimental animals and holding conditions

Juvenile rainbow trout (6 – 10 g) were purchased from Humber Spring Trout Hatchery (Orangeville, Ontario), and held in batches of 50 fish per 200-L aerated aquaria, supplied with dechlorinated Hamilton tap water (12^{o}C), pH ~ 7.5, flow rate ~ 1 L min^{-1}, photoperiod of 12.5 h light: 11.5 h dark, at McMaster University for 3 weeks prior to experimentation. Fish were fed a 1% total tank weight ration with Martin's commercial dried pellet feed (1 point; Martin Mills Inc., Elmira, Ontario) three times per week. Water composition was: (in mmol L^{-1}) Na^+ = 0.5, Cl^- = 0.7, Ca = 1.0, hardness ~140ppm as $CaCO_3$.

2.2. Hierarchy preparation

Fish were anaesthetized individually in neutralized MS-222 (0.08 g tricaine methanesulfonate L^{-1}), weighed (0.01g), and fork length (0.1 cm) was measured. Each fish was uniquely freeze branded to allow for visual identification. This was achieved using a surgical probe dipped into liquid nitrogen: the cold tip was pressed behind the head to form a distinctive mark. Fish were air-exposed for no more than 1 min and regained normal behaviour after a day, with feeding occurring two days after anaesthetization. No severe side-effects were observed. Four sized-matched fish were then placed inside an aerated white 30-L tank (53 x 26.7 x 30 cm) supplied with flowing water (water quality the same as holding conditions, though in-tank measurements of pH

were routinely 7.2-7.4). A clear lid facilitated behavioural observations. Five pieces of PVC pipe (1 floating) (7 x 2.5 cm) were also added to the tank to serve as shelter.

2.3. Hierarchical creation and feeding regime

To create social hierarchies and record physiological differences between fish, a new technique was designed, using a darkened container. Fish were fed using a darkened, plastic container (17.8 x 14 x 12 cm, volume = ~ 2.8-L) (Fig. 1) with a feeding tube attached to it, so that the food pellets were deposited inside the container. Food was therefore highly localized in one zone inside the darkened container. Fish associated the darkened feeding container with food and attempted to monopolize the feeding area.

Using this method, fish were placed on a strict feeding regime, consisting of two feedings daily of 1% total tank biomass (morning between 7:30-9:00 AM and evening between 6:30-8:00 PM). Food was delivered pellet by pellet into the feeding tube, after which a small amount of tank water (collected with a small beaker) was used to push the left-over pellets into the feeding container. Dispensing food pellets took less than a minute. The feeding container was left inside the tank for 15 min (during which a video camera recorded behaviour) for 10 consecutive days. The first 5 days are termed as period 1, with day 6-10 referred to as period 2. Data were obtained from a total of 7 separate hierarchies, all set up in an identical fashion.

2.4. Behavioural measurements

Morning feedings were selected as the behavioural study period because preliminary results showed higher aggression in the morning compared to evening feedings. During morning feedings, a video camera (Sanyo VPC-WH1, Osaka, Japan) was set up on scaffolding surrounding the tank to videotape aggressive behaviour for 15 minutes. Only behaviour outside the feeding container could be recorded since the feeding container was dark. Individual feeding could not be determined from video data. Aggressive behaviour was also monitored during non-feeding periods. Aggressive behaviour was similar in both quantity and intensity between morning feedings and afternoon periods when the feeding container was absent, so only data from the morning feeding periods was utilized.

Each aggressive act (chase or approach which caused the other fish to react) was scored 1 point, allowing for a dominance hierarchy to be recorded, with dominant individuals having higher scores than subordinate individuals. Therefore, rank 1 fish were the most dominant and rank 4 were the least dominant.

2.5. Physiological measurements

Fish were starved for 24 hours prior to physiological measurements, which were recorded at the ends of each of the two periods (on day 5 and day 10 of the experiment). In order to confine individual fish, a 'dummy' feeding apparatus (water-tight and air-tight), identical in appearance to the darkened feeding container, was inserted into the tank. Fish would enter, presumably assuming that food would be located inside. The lid

was then closed, trapping the fish inside and the 'dummy' feeding container was placed in a water bath at $12^{O}C$. From here, water samples were taken at hourly intervals for 6 hours without disturbing the fish.

Oxygen consumption was calculated over the first hour during which the fish was held in the 'dummy' container. Water samples were analyzed using an oxygen electrode (Cameron Instrument, Port Aransas, Texas) thermostatted to the experimental temperature ($12^{0}C$) and connected to a Model 1900 A-M Systems Polarographic Amplifier digital dissolved oxygen meter (Carlsborg, Washington). After the first hour of holding, the water in the 'dummy' feeding container was gently aerated, and aeration continued for the next 5 hours so as to maintain air saturation. This longer time period was required to obtain an accurate ammonia flux measurement (assay procedure modified from Verdouw et al.,1978). At the end of the 6 hours, fish were individually anaesthetized in MS-222 (0.08 g L^{-1}), weighed, measured for fork length, and allowed to recover in their respective tanks.

The methodology of McCarthy et al. (1992) was used to determine individual food consumption. On the morning of day 11, fish were fed a ration of 1% total tank weight of repelleted Martin commercial pellet feed (see Alves et al. (2006) for detailed description) containing 6% (by mass of dry powered food) Ballotini lead glass beads (0.400 to 0.455-mm; 8.5-grade, Jencons USA, Inc., Bridgeville, Pennsylvania). One hour after feeding, fish were terminally sampled with a concentrated dosed of neutralized MS-222 (5 g L^{-1}) to cause quick euthanization without struggling. Fish carcasses were frozen

at -20^{0}C until X-rayed (Faxitron 805 portable X-ray machine, Wheeling, Illinois; 1 second exposure at 70 kVP) to determine the number of glass beads ingested.

2.6. Calculations

During each morning feeding (8 in total – days 1 though 4 and day 6 through 9), all fish were scored for aggressive behaviour. Aggressive acts were not scored on day 5 and day 10 as physiological measurements were conducted. Total aggressive acts for each fish were then divided by the 8 days of observations, to yield aggressive acts per day (each day representing a 15-min observation period).

To determine individual food consumption of a single meal, a conversion from glass beads to food consumption was accomplished by averaging the number of beads per pellet (see McCarthy et al., 1992, for detailed description), and counting the beads in each fish as a percentage of the total number of beads recovered.

Specific growth rate (SGR) was calculated as:

1. $(\ln(BM_2)-\ln(BM_1))/(t_2-t_1) \times 100$,

where BM_1 and BM_2 were body masses at times t_1 and t_2, respectively.

Fulton's condition factor was calculated as:

2. $(BM_{(g)}/L^3_{(cm)})*100$,

where BM is the weight and L is the length of the fish. Percent change in condition factor was calculated from beginning of the experiment to the end. A positive

percent change indicates increasing condition factor from beginning to end of the experiment.

Oxygen consumption (MO_2) was calculated as follows:

3. $(\Delta PO_2 \times \alpha O_2 \times v)/(m \times t)$,

where ΔPO_2 (mmHg) is the measured change in PO_2 values between beginning and end of the first hour of physiological testing,

αO_2 ($\mu mol\ L^{-1}\ mmHg^{-1}$) is the solubility constant for O_2 in water (Boutilier et al. 1984),

v is the volume (L) of the 'dummy' feeding container,

m (g) is the mass of the fish, and t is the time (h).

A similar equation was used to calculate ammonia excretion, substituting total ammonia – N for $\Delta PO_2 \times \alpha O_2$.

To calculate protein utilization, instantaneous relative use of protein as an aerobic metabolic fuel, the nitrogen quotient (NQ) was first calculated as outlined by Lauff and Wood (1996).

4. M_{Ntotal}/MO_2.

Protein utilization was then determined as:

5. NQ/0.27,

where 0.27 is the theoretical maximum for NQ in a teleost fish where 100% of aerobic metabolism is fueled by proteins (and amino acids) as derived by van den Thillart and Kesbeke (1978).

2.7. Statistical analyses

Statistical analyses were conducted using both SigmaStat 3.5 (Systat Software, Inc. 2006) and Statistica 7.0 (StatSoft Inc. 2004) software. Data have been analysed in two different ways: 1) where physiological parameters were averaged on the basis of the fish's social status (i.e rank in the hierarchy) and 2) where physiological parameters were plotted against the measured aggressive behaviour of the individual.

2.7.1. Social status and physiological parameters

One-way repeated measures ANOVA followed by the Holm-Sidak post-hoc test were performed to test for differences in oxygen consumption, ammonia excretion, and protein utilization between the four ranks of social status. Oxygen consumption data was log transformed to pass normality tests. Differences between growth and condition factor among the four ranks of social status were tested by One-way ANOVA followed by the Holm-Sidak post-hoc test. Non-parametric Kruskal-Wallis one-way ANOVA was performed followed by Holm-Sidak post-hoc test to test for differences in aggressive acts per day and feeding among the four ranks of social status. Each social status rank contained seven individuals, one from each experimental hierarchy (N=7), and data were expressed as means ± 1 SEM.

2.7.2. Individual behaviour and associated physiological parameters of the individual

Measures of individual behaviour and resulting physiology for each fish in each of the 7 experimental hierarchies (total N=28 individuals) were analysed by the Spearman rank correlation. Scatter plots of individual feeding, growth, percent change in condition factor, oxygen consumption, ammonia excretion, protein utilization and aggressive acts per day were created. Significance was assigned based on rejecting the null hypothesis.

Probability levels have been reported throughout, and $P \leq 0.05$ was considered significant.

3. Results

3.1. Behavioural measurements

To observe activity inside the feeding container during a feeding, a clear container (exactly the same as the darkened feeding container except the coloration had been removed) was used to feed two different hierarchies (a stable hierarchy was already established in each case). Feeding occurred normally, compared to the darkened feeding container, with no aggressive acts taking place inside the clear feeding container. The dominant fish consumed the majority of the food with the other social ranked fish entering sporadically to retrieve what was left over. At times, two fish cohabitated the feeding container at the same time with the less dominant fish simply leaving randomly.

Dominance hierarchies were formed in all experimental groups over the course of the experiment, with six of the seven establishing stable hierarchies (consistent aggressive acts over 2 days) in period 1. One tank needed eight days of observation before a consistent rank could be determined. It was in this experimental group where the only observed rank 'switch' occurred: rank 1 and rank 2 fish swapped social status after period 1, becoming rank 2 and rank 1, respectively, in period 2.

Overall, rank 1 fish had significantly more aggressive acts (12.5 aggressive acts per day) than rank 2 (5.7 aggressive acts per day), 3 (1.5 aggressive acts per day) or 4 (0.4 aggressive acts per day) (Fig. 2). Ranks 2 and 3 also differed significantly. In decreasing order, rank 1 exhibited 62.5%, rank 2 showed 28.3%, rank 3 displayed 7.3% and rank 4 accounted for only 1.9% of aggressive acts per day.

Aggressive acts per day per period for each hierarchy did not differ between period 1 and period 2 (21.9 ± 4.2 and 18.2 ± 2.5 aggressive acts per day, respectively for period 1 and period 2). Three hierarchies displayed more aggressive acts in period 1 than in period 2 (25.7, 40.5, and 31.2 compared to 16.0, 18.2 and 9.0 aggressive acts per day). However, in the other four hierarchies the opposite was observed (14.0, 9.8, 14.7, and 17.5 during period 1 compared to 30.0, 15.5, 16.5, and 22.2 aggressive acts per day in period 2). There was also no statistical difference between total aggressive acts per hierarchy, with aggressive acts per hierarchy ranging from 12.6 to 29.3 per day (mean 20.0 ± 2.0).

3.2. Physiological measurements

3.2.1. Feeding

Dominant rainbow trout (rank 1) consumed the most food, eating 66.9 ± 7.0% of the total meal (Fig. 3A). Subordinate rainbow trout (rank 2, 3 and 4) consumed significantly lower amounts, 23.2 ± 9.2, 5.7 ± 3.4 and 4.2 ± 2.7%. There was no significant difference among ranks 2, 3 and 4.

A highly significant positive correlation between feeding percentage and aggressive acts per day existed in individual fish (Fig. 3B). Some fish with low aggressive acts did not consume any food, while fish with high aggressive acts consumed more food, regardless of rank.

3.2.2. Growth

Rank 1 fish had significantly higher specific growth rates than rank 2, 3, and 4 fish, with no difference being observed among the three subordinate ranks (Fig. 4A). Rank 1 fish had a specific growth rate of 2.49 ± 0.37 %day^{-1} compared to 0.58 ± 0.39, 0.02 ± 0.29 and -0.27 ± 0.32 %day^{-1} for ranks 2, 3 and 4, respectively. There was no relationship between starting weight or length and final social status.

Individual fish with higher aggressive acts had higher specific growth rates compared with fish displaying lower aggressive acts, regardless of social status. A positive and highly significant correlation existed between specific growth rate and aggressive acts in individual trout (Fig. 4B).

3.2.3. Condition factor

Percent change in condition factor was significantly higher in rank 1 compared to other ranks (21.9 ± 4.1 %). Ranks 2 and 3 had similar percent changes in condition factor (4.1 ± 3.9 and 2.4 ± 3.6 %, respectively) with rank 4 having the lowest (-0.5 ± 3.7%) (Fig. 5A). There was no significant difference among subordinate ranked fish (ranks 2, 3 and 4). There was no correlation between condition factor at the beginning of the experiment and final social status.

A strong positive correlation was observed between percent change in condition factor and aggressive acts per day (Fig. 5B). Fish tending to have high aggressive acts per

day also exhibited high positive percent changes in condition factor compared to fish showing low aggressive acts.

3.2.4. Oxygen consumption

Rank 1 had the lowest oxygen consumption, 13.39 ± 2.02 µmol-O_2 $g^{-1}h^{-1}$, while ranks 2, 3 and 4 had similar oxygen consumptions: 22.61 ± 2.07, 23.92 ± 4.69, and 23.22 ± 7.09 µmol-O_2 $g^{-1}h^{-1}$, respectively (Fig. 6A). There was no significant difference among the four ranks. However, when all subordinate ranks (rank 2, 3 and 4) are combined and compared to rank 1 fish, rank 1 consumed a significant lower amount of oxygen than the combined subordinates (Mann-Whitney U Statistic, p=0.017).

There was also no significant correlation between oxygen consumption and aggressive acts per day in individual fish (Fig.6B). Note that subordinate fish exhibited more variable MO_2 values.

3.2.5. Ammonia excretion

Ammonia excretion was the highest in rank 1 fish, with rates of 1.43 ± 0.22 µmol-N $g^{-1}h^{-1}$ compared to 0.85 ± 0.11, 0.99 ± 0.13 and 0.73 ± 0.11 µmol-$ng^{-1}h^{-1}$ in ranks 2, 3 and 4, respectively (Fig. 7A). There was no significant difference among ranks 2, 3 and 4.

A significant positive correlation was observed between ammonia excretion and aggressive acts per day in individual fish (Fig. 7B), with fish displaying high aggressive

acts tending to have higher ammonia excretion rates compared to fish that exhibited low aggressive acts.

3.2.6. Protein utilization

Rank 1 had the highest percent protein utilization in aerobic metabolism (48.7 ± 8.9%), a value that was significantly higher than all the other ranks. Percent protein utilization was similar among ranks 2, 3 and 4 fish: 15.5 ± 2.1%, 26.4 ± 8.3% and 17.5 ± 4.1%, respectively (Fig. 8A). However, there was no significant difference among ranks 2, 3 and 4.

Percent protein utilization exhibited a significant positive correlation with aggressive acts per day in individual trout (Fig. 8B). Fish displaying lower aggressive acts tended to have lower percent protein utilization compared with those displaying higher aggressive acts.

4. Discussion

4.1. Behaviour

Aggression was the only behavioural measurement necessary to assign social status to individual fish, as fish in each hierarchy displayed adequate levels of aggressive acts to create a linear dominance hierarchy. All groups of fish formed social hierarchies within 8 days of feeding, which is consistent with the length of most dominance studies in fish (see Sloman and Armstrong, 2002, for a comprehensive review on the duration of assessing hierarchies).

The level of aggression per hierarchy did not differ between periods 1 and period 2 (Section 3.1). This is not a surprising finding. Brown trout in hierarchical groups of 4 have also been documented to exhibit consistent daily aggression for the duration of a 2-week study (Sloman et al., 2000a). However, it has been reported that daily aggression decreased in salmon and rainbow trout (Brown and Brown, 1993) while another report suggested aggression increasing in zebrafish hierarchies from the beginning to the end of experiment (Filby et al., 2010). Individual aggression can be modified quickly based on current circumstances (Ward et al., 2006) which is influenced by genetics (Van Leeuwen et al, 2011).

However, a possible explanation for constant aggression level in the present study *versus* the changing aggression levels in the aforementioned studies could be differences in the time schedule of feeding and behavioural measurements. In the present study and Sloman et al. (2000a), behavioural measurements were conducted at the start of feeding,

while Brown and Brown (1993) and Filby et al. (2010) both videotaped behaviour before feeding occurred. This suggests that there might be a limit for total or consistent aggression to take place during a feeding event. During feeding, fish not only have to out-compete others to gain access to food, they also have to consume the food. In hierarchies created in the studies by Brown and Brown (1993) and Filby et al. (2010) fish were fighting for position to feed and thus could allocate as much time and energy to aggression as needed. Few studies report aggression levels throughout their experiment and thus definitive conclusions are difficult to draw.

4.2. Physiology

When the physiological data from the 7 different hierarchies were grouped as to rank, there appeared to be two 'classes' of physiologically different fish amongst the four fish in each hierarchy: dominant individuals that consumed a higher percentage of food, had higher growth rate, higher percent change in condition factor, higher ammonia excretion, higher protein utilization and lower routine metabolic rate (i.e. O_2 consumption) compared to the three other fish that became subordinate individuals and had similar physiology to each other. This would suggest the dominant fish being favoured in such a competitive setting. However, when individual behaviour was taken into account, a linear correlation was observed between aggressive acts and most measures of the resulting physiology, suggesting a continuum of physiological responses related to aggression levels. While this finding is not innately novel, many studies report

either ranked or individual data. The present study is the first, to the author's knowledge, to compare grouped social status with individual aggression. The implications of the two different methods of data analysis are explored in the following Section 4.3.

Dominant fish used aggression to monopolize food, restricting the consumption of other fish (Fig. 3A), which is in agreement with previous studies (Nicieza and Metcalfe, 1999; Sloman et al., 2001). These studies, including the present, suggest that monopolizing food has an effect on growth rate, with dominant individuals displaying higher growth rates compared to subordinates (Fig. 4A). However, it is not known if dominant fish consumed the majority of food every day, since feeding consumption was only measured once, at the end of the 10-day experiments.

Higher percent change in condition factor was observed in dominant relative to subordinate fish and may also be attributed to feeding (Fig. 5A). Condition factor, which can be used as an index for fish health (Gilmour et al., 2005), can be influenced by food consumption. Fish with high food consumption can have energy reserves and repair damage, and as consequence be healthier. Gregory and Wood (1999) reported that trout which consumed a higher ration in a hierarchy had higher (healthier) fin condition index compared to individuals who ate less.

A similar study to the present one, involving hierarchies of four brown trout, was conducted by Sloman et al. (2000b) and reported a different result with regards to change in condition factor. Rank 2 individuals had a negative change in the residual condition factor, while rank 1, 3 and 4 had positive, non-significant change in condition factor. It is

not known why this difference exists between these two studies, but it could possibly be attributed to the relatively complex semi-natural stream environment used by Sloman et al. (2000b) compared to a the more uniform laboratory setting of the present investigation.

Low routine metabolic rate was observed in dominant fish (see Section 3.2.4.). This falls in-line with past research done by Sloman et al. (2000c) where dominant individuals had lower standard metabolic rate (SMR) than subordinates after being confined in pairs. Dominant individuals are expending less energy than the subordinates while consuming the highest percentage of food. This means there are energy stores which can be used for growth and repair, correlating with observed higher growth rates and higher percent change in condition factor than the subordinates. Clearly, dominant individuals are being favored in this laboratory setting.

However, in a recent study it was shown that quantity of food consumption had a direct effect on standard metabolic rate in juvenile coho salmon (Van Leeuwen et al., 2011). Higher food consumption caused an elevation in SMR. This was not observed in the current study as dominant individuals monopolized food but had lower oxygen consumption rates compared to subordinates who consumed less food but did have higher metabolic rate. The main difference between the two studies was that coho salmon were not held in dominance hierarchies, where stress could be a factor. In the present study, dominant individuals are being compared to subordinates, who might have elevated plasma cortisol (discussed below).

Cortisol could play a role in oxygen consumption as it has been established that subordinate individuals in laboratory settings may have elevated plasma cortisol levels (Fox et al., 1997; Sloman et al., 2001) which could cause increased oxygen consumption in trout (Morgan and Iwama, 1996; Sloman et al., 2000c; De Boeck et al., 2001). Cortisol is potentially the reason for the observed difference seen in the current study and that of Sloman et al. (2000c), in comparison to the results of Van Leeuwen et al. (2011). However, since cortisol was not collected during physiological measurements in the present investigation, cortisol's effect on oxygen consumption in this study is anecdotal. In a parallel study conducted under similar circumstances (reported in Chapter 3), subordinate individuals tended to have elevated, but not significantly different, plasma cortisol levels compared to dominant fish. This suggests that cortisol could have played a role in the higher oxygen consumption rates that were observed in the current study.

Differences in food consumption and metabolism may also explain the differences seen in ammonia excretion and protein utilization between the dominant fish and the rest of the subordinates. Rank 1 fish that consumed the most food and had the lowest metabolic rate also had the highest ammonia excretion rate and protein utilization, while ranks 2, 3, and 4 displayed similar ammonia excretion and protein utilization while having similar food consumption and oxygen consumption (Fig. 7A and Fig. 8A). It has been previously shown that fish fed to satiation did have higher ammonia excretion rates compared to fish that were not fed (Alsop and Wood, 1997; Bucking and Wood, 2008) but these measurements were taken within 12-h of feeding. In the present study,

physiological parameters were taken at 24 h+ post-feeding, when the animal should be in a post-absorptive state (Brett and Zala, 1975). Therefore, it appears that dominant individuals in the present hierarchies were burning more muscle protein for energy. An additional cause could be increased ATP turnover and associated degradation, because Mommsen and Hochachka (1988) showed that working muscle in rainbow trout produces ammonia through deamination of adenylates. This suggests that dominant individuals are not as efficient as they could be, using muscle tissue as a fuel source. This is possibly a necessary trade-off, between maintaining a high social status within the hierarchy and building muscle.

4.3 Implications of two different methods of data analysis

Two physiologically distinct groups were observed when the data were grouped as to rank of social status in dominance hierarchies consisting of four rainbow trout in a laboratory setting. However, investigating the individual behaviour produced a positive, linear correlation between most physiological parameters and aggressive acts per day. This suggests that there are two possible methods for interpreting a social hierarchy, which can lead to different conclusions.

While not incorrect, grouping individuals from different hierarchies together based on their respective social status can produce inherent problems. Each hierarchy consists of a different set of fish and thus each hierarchy will be different. To account for this, Briffa and Elwood (2010) suggest using repeated measures statistics to analyse the

group data, and indeed this was the method used in the present study. This approach is valid as far as the statistics are concerned as it measures differences between ranks taking into account different experimental groups (i.e. different hierarchies). But as the present study illustrates, representing hierarchies based on social status does create a concern that needs to be addressed.

The concern lies in 'hiding' the variability of both physiological and behavioural measurements. This is due to individual behaviour affecting physiology. Since each hierarchy consists of different fish, each hierarchy will inevitably have a different behavioural environment. Each fish will then adapt its behaviour based on its unique environment. For example, in a high aggression hierarchy, rank 4 fish might perform 2 aggressive acts per day, while a rank 4 fish in less competitive hierarchy might display 0 aggressive acts or vice versa. The two fish are in different environments and are thus behaving differently with different resulting physiology. However, when social status is assigned these fish are grouped together. This creates a potential "statistical artifact" and information content on individual behavior is masked.

However, when observing aggressive acts per day, a linear correlation exists between many physiological measures and behaviour regardless of assigned social status (Figs. 3B, 4B, 5B, 7B, 8B). As aggression per individual increases so does the resulting physiology, and the pattern represents a continuum. This conclusion is 'hidden' in the social status data.

To further illustrate that aggression per fish reveals a more complete story with regards to the physiological state of fish inside a hierarchy, aggression levels per hierarchy were standardized by calculating the mean aggressive acts per hierarchy and measuring the difference of each fish to that mean (i.e. a positive value indicating that fish performed more aggressive acts than the mean in their respective hierarchy, and *vice versa*). As seen in the example of Fig. 9, in comparison with Fig. 4, a stronger correlation exists between individual aggressive acts and specific growth rate when the level of aggression in each hierarchy is controlled (R=0.824 versus R=0.677). Similar differences (i.e. stronger linear correlations) were seen when the same analytical approach was applied to feeding, condition factor, oxygen consumption, ammonia excretion and protein utilization (data not shown). This shows that physiology of fish in a dominance hierarchy is affected by the individual's specific behaviour in response to its unique behavioural environment.

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Figure 1. Diagram of darkened feeding container (17.8 l x 14 h x 12 cm w, volume = 2.8-L). Food pellets were dropped into the darkened container via the feeding tube, where food was localized inside. To capture fish, a 'dummy' feeding container was placed inside the tank and as soon as a fish entered the lid was closed, confining the fish inside.

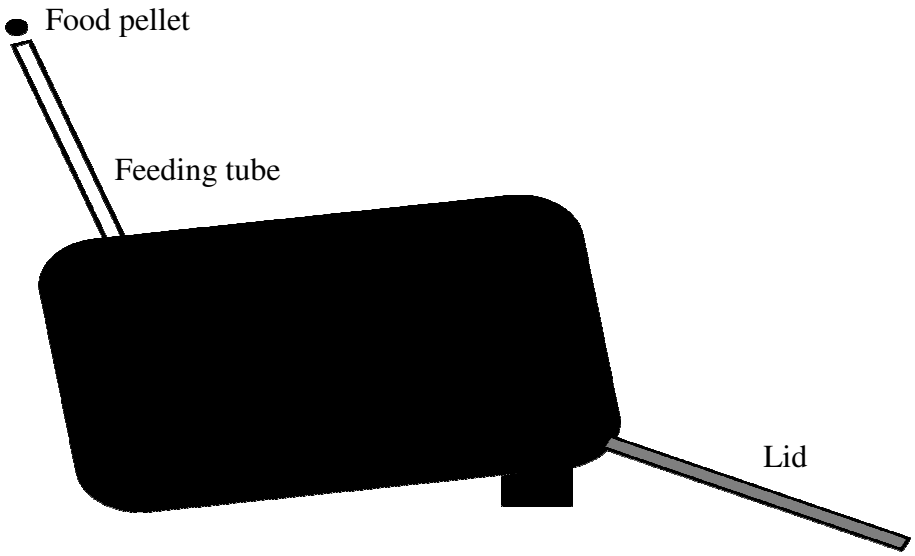


Figure 1

Figure 2. Aggressive acts per day based on social status. Values are means ± S.E.M.: N=7 experimental groups. Different letters denote significant differences among ranks (p=<0.001; Kruskal-Wallis one-way ANOVA; post-hoc test Holm-Sidak).

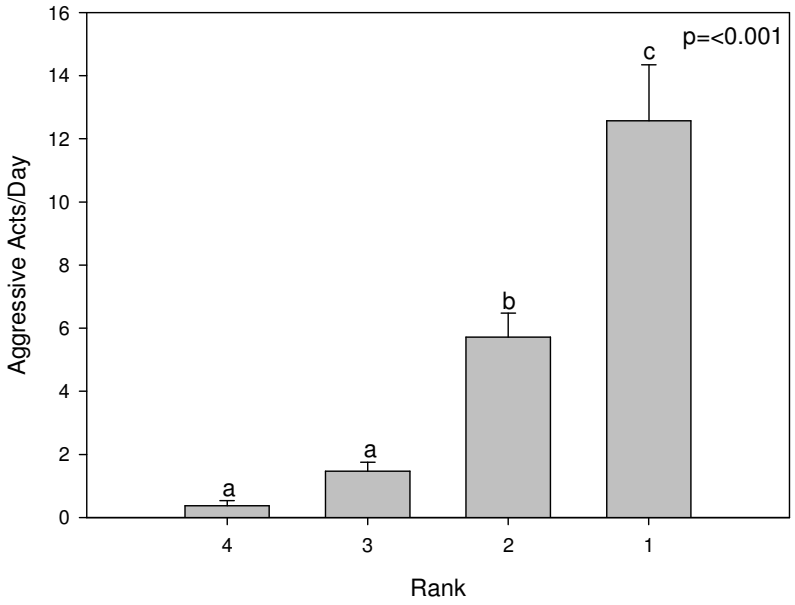


Figure 2

Figure 3. A & B. A. Percent feeding for one meal based on social status. Values are means ± S.E.M.: N= 7 experimental groups. Different letters denote significant differences among ranks (p=<0.001; Kruskal-Wallis one-way ANOVA; post-hoc test Holm-Sidak).B. Relationship between percent feeding of one meal and aggression per day. Percent feeding for one meal and averaged total aggressive acts (N=28; Spearman Rank Correlation; Rs=0.631; p=0.000).

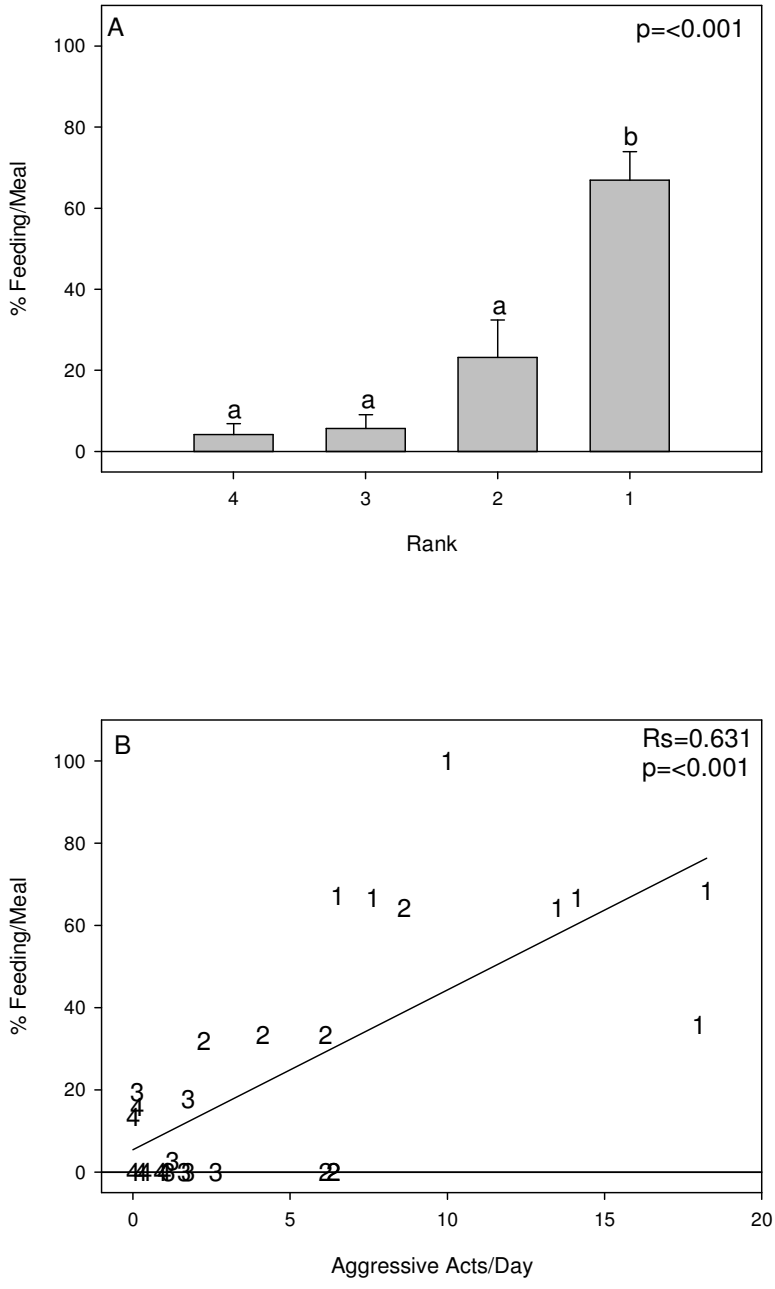

A
p=<0.001
% Feeding/Meal
100
80
60
40
20
0
a
a
a
b
4
3
2
1
Rank
B
Rs=0.631
p=<0.001
% Feeding/Meal
Aggressive Acts/Day
0
5
10
15
20

Figure 3 A & B

Figure 4. A & B. A. Specific growth rate per day based on social status. Values are means ± S.E.M.: N= 7 experimental groups. Different letters denote significant differences in specific growth rates per day among ranks (p=<0.001; One-way ANOVA; post-hoc test Holm-Sidak). B. Relationship between specific growth rate and aggression per day. Specific growth rate and averaged total aggressive acts. N=28 (Spearman Rank Correlation, Rs=0.677, p=0.000).

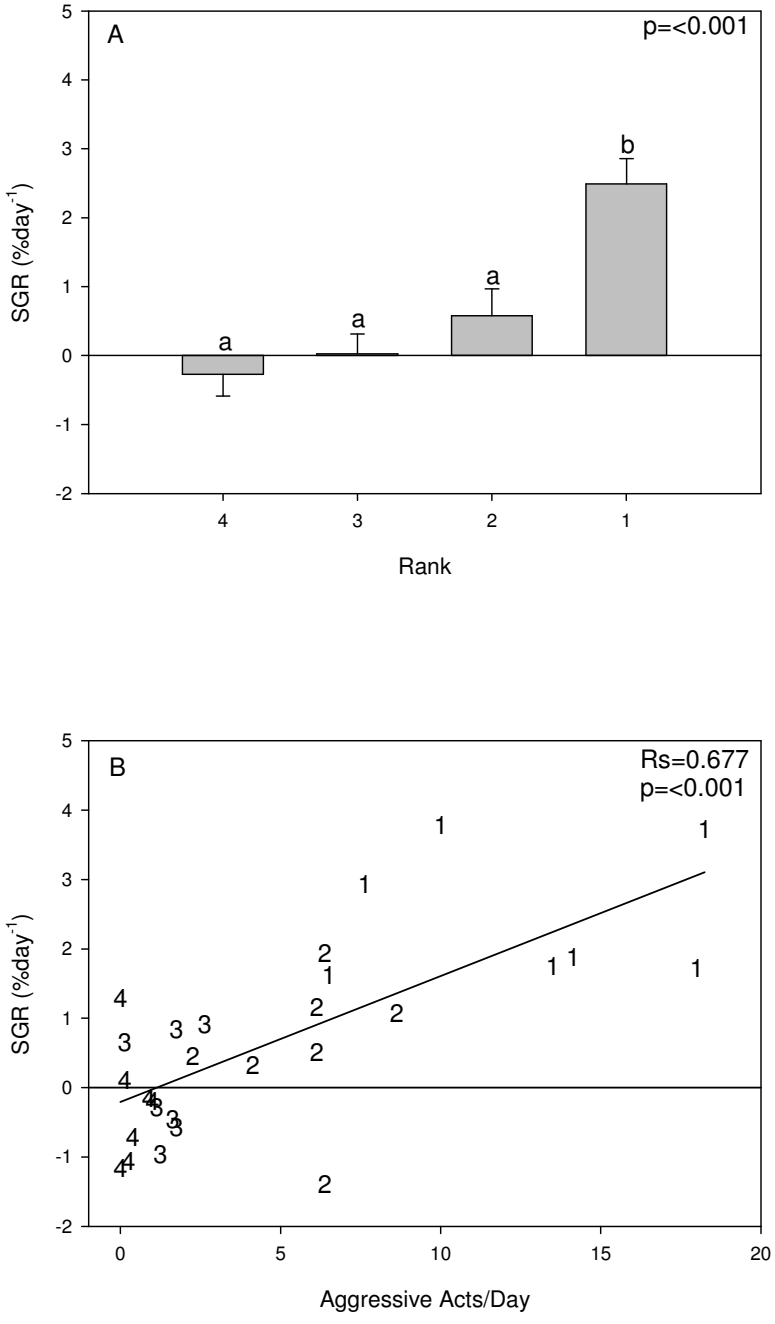

A
p=<0.001
SGR (%day-1)
Rank
a
a
a
b
B
Rs=0.677
p=<0.001
SGR (%day-1)
Aggressive Acts/Day

Figure 4 A & B

Figure 5. A & B. A. Percent change in condition factor based on social status. Different letters signify statistical differences among ranks. Values are means of ± S.E.M.: N=7 experimental groups (p=0.001; One-way ANOVA; post-hoc test Holm-Sidak). B. Correlation between percent change in condition factor and aggression per day. Percent change in condition factor from start of the experiment to the end of period 2 (N=27; Spearman Rank Correlation; Rs=0.501; p=0.007).

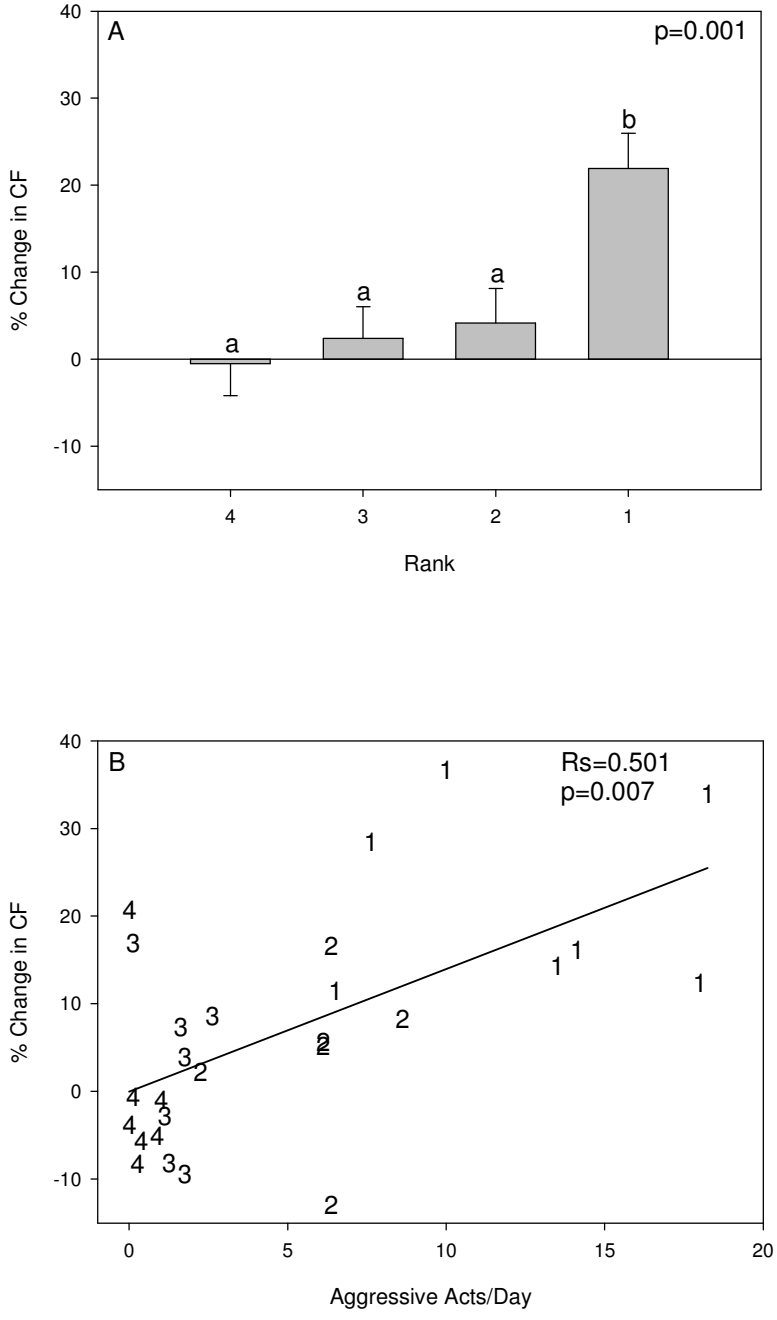

A
p=0.001
% Change in CF
Rank
a
a
a
b
B
Rs=0.501
p=0.007
% Change in CF
Aggressive Acts/Day

Figure 5 A & B

Figure 6. A & B. A. Oxygen consumption rates based on social status. Values are means ± S.E.M.: N= 7 experimental groups (p=0.073; Repeated measures one-way ANOVA; post-hoc test Holm-Sidak). B. Oxygen consumption and aggression per day. Oxygen consumption and averaged total aggressive acts (N=28; Spearman Rank Correlation; Rs=-0.292; p=0.130).

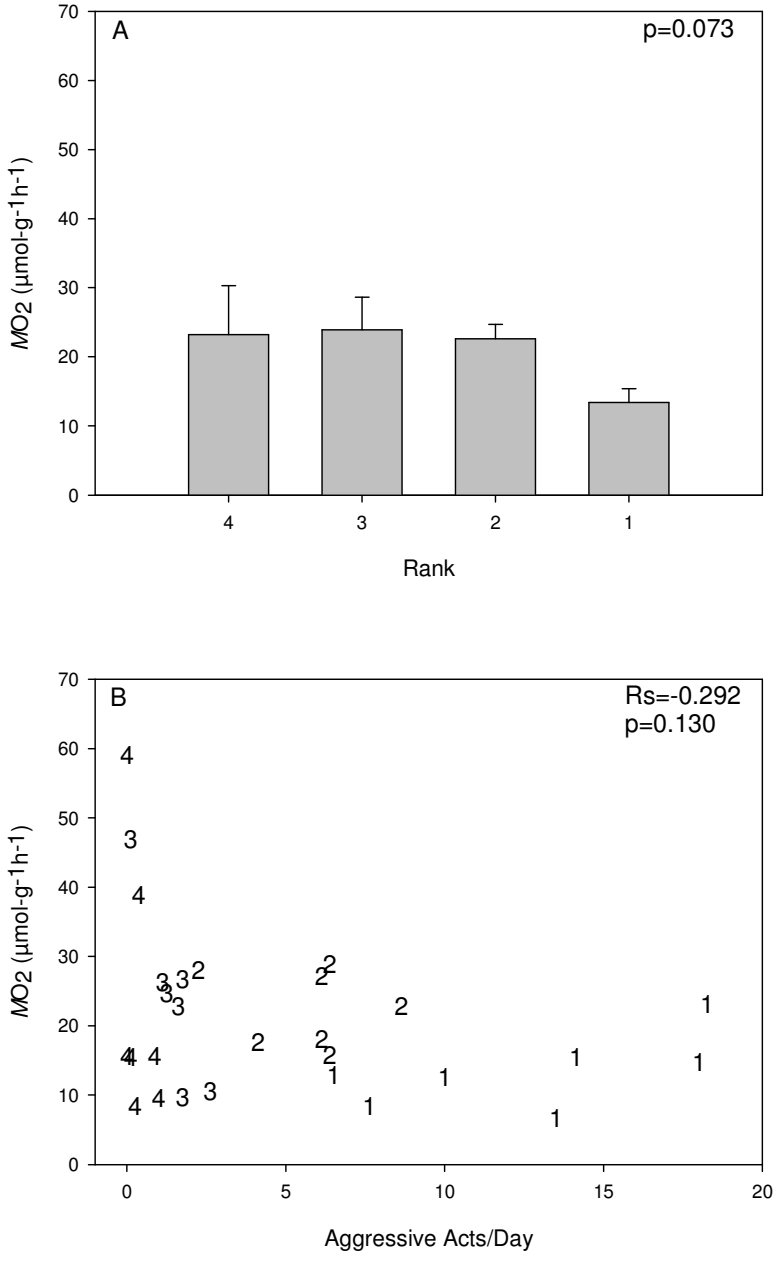

A
p=0.073
MO2 (µmol-g-1h-1)
Rank
B
Rs=-0.292
p=0.130
MO2 (µmol-g-1h-1)
Aggressive Acts/Day

Figure 6 A & B

Figure 7. A & B. A. Ammonia excretion rates based on social status. Different letters signify statistical differences among ranks. Values are means ± S.E.M.: N= 7 experimental groups (p=0.018; Repeated measures one-way ANOVA; post-hoc test Holm-Sidak). B. Correlation between ammonia excretion and aggression per day. Ammonia excretion and averaged total aggressive acts (N=28; Spearman Rank Correlation; Rs=0.424; p=0.024).

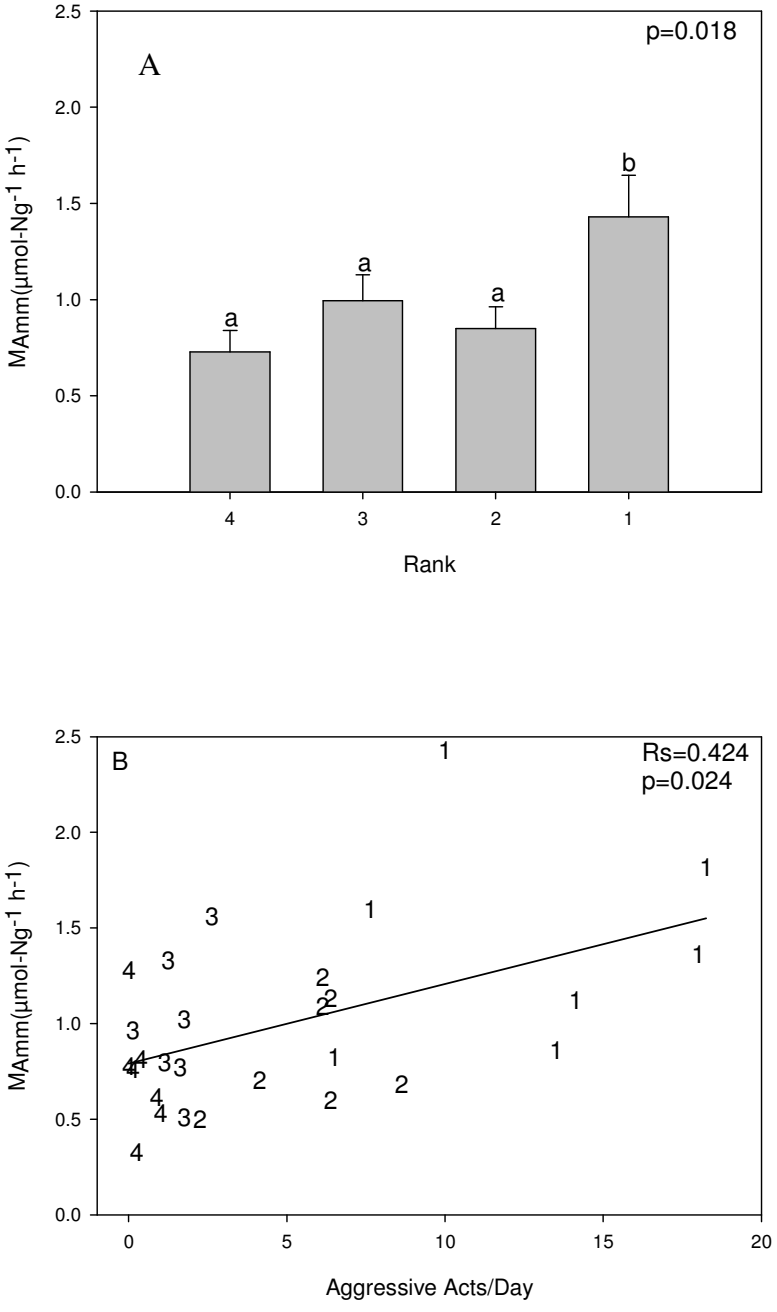
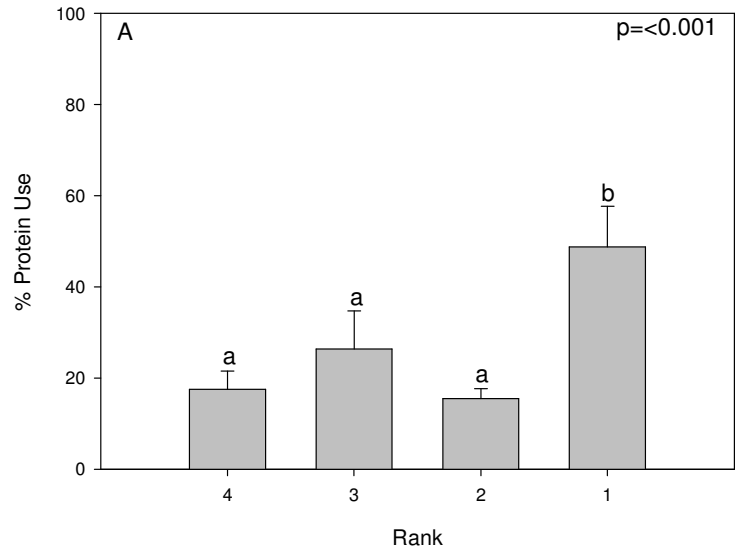

A
p=0.018
MAmm(µmol-Ng-1 h-1)
Rank
a
a
a
b
B
Rs=0.424
p=0.024
MAmm(µmol-Ng-1 h-1)
Aggressive Acts/Day

Figure 7 A & B

Figure 8. A & B. A. Percent protein use based on social status. Different letters signify statistical differences among ranks. Values are means of ± S.E.M.: N=7 experimental groups (p=<0.001; Repeated measures one-way ANOVA; post-hoc test Holm-Sidak). B. Correlation between protein use and aggression per day. Percent protein use and averaged total aggressive acts (N=27; Spearman Rank Correlation; Rs=0.415; p=0.031).


A
p=<0.001
% Protein Use
Rank
a
a
a
b
100
80
60
40
20
0
4
3
2
1

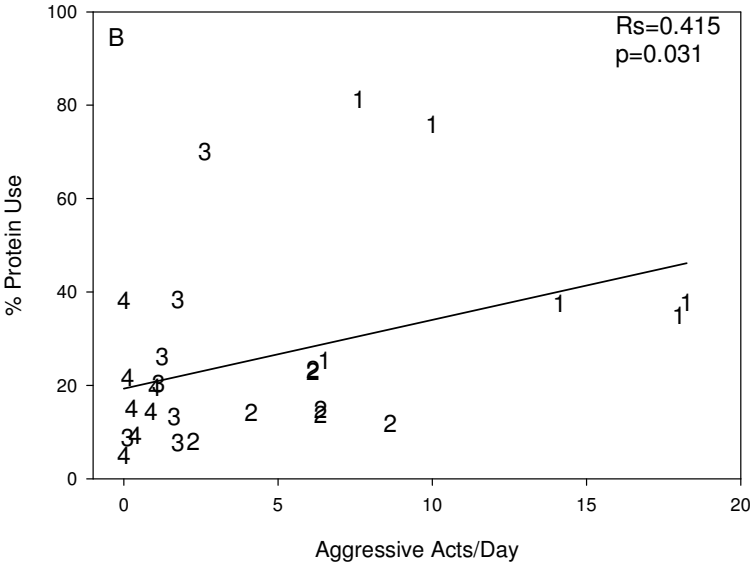

B
Rs=0.415
p=0.031
% Protein Use
Aggressive Acts/Day
100
80
60
40
20
0
0
5
10
15
20

Figure 8 A & B

Figure 9. Relationship between specific growth rate and net aggressive acts different from hierarchy aggressive mean. Mean aggressive acts for each hierarchy was calculated. Next, aggressive acts performed by each fish was subtracted from their respective hierarchical mean. Positive values indicating fish displayed more aggressive acts than the hierarchical mean and negative values representing fish that displayed less aggression than their hierarchical mean. (N=28, Spearman Rank Correlation, Rs=0.824, p=0.000).

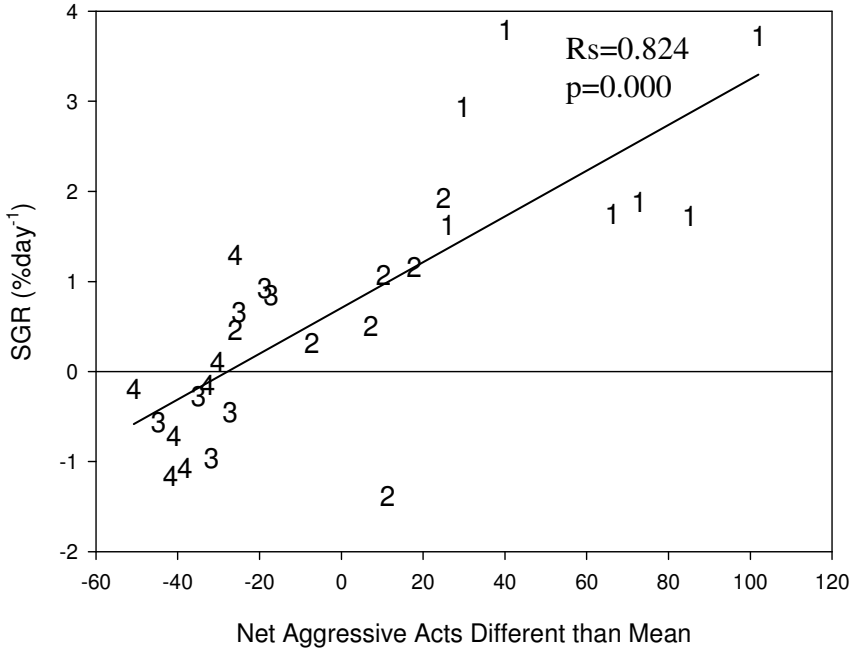

Rs=0.824
p=0.000
SGR (%day-1)
Net Aggressive Acts Different than Mean
4
3
2
1
0
-1
-2
-60
-40
-20
0
20
40
60
80
100
120

Figure 9.

Chapter 3: High external ammonia's effect on the formation of rainbow trout hierarchies and resulting physiological changes

Abstract

Ammonia can enter the aquatic environment through natural and anthropogenic sources, impacting ionoregulation, swimming performance, appetite and growth in fish. However, the effects of elevated ammonia concentrations on the formation of dominance hierarchies, or the physiology of fish therein, has not been investigated. The objectives of this study on juvenile rainbow trout (*Oncorhynchus mykiss)*, were twofold: firstly, to determine whether sublethal concentrations of water-borne ammonia would prevent the formation of a hierarchy in groups of four trout or alter its structure; and secondly, to investigate the behavioural and physiological changes of individuals exposed to a high concentration of ammonia that does allow hierarchical formation. Experimental ammonia concentrations were: 700, 1200 and 1500 µM total ammonia (or 2.97, 5.10, 6.37 µM NH_3, respectively) at pH 7.2 (12^oC), which are above allowable water quality guideline values for chronic ammonia exposures. Hierarchies were established as outlined in Chapter 2 and were exposed to elevated ammonia (NH_4HCO_3) 12 hours before first feeding. The concentrations were maintained for the duration of the 11-day experiment, with parallel ammonia-free controls. Aggression was recorded by video camera during morning feedings. In the first set of experiments, 1200 or 1500 µM total ammonia severely reduced aggressive behaviour, such that social hierarchies could not be determined, with no aggression being documented in the groups of fish exposed to 1500

μM total ammonia. For the second set of experiments, groups of trout were exposed to 700 μM total ammonia for 11 days, with physiological parameters recorded on day 5 (end of period 1) and day 10 (end of period 2), as in Chapter 2; feeding and plasma cortisol were measured on day 11. Fish exposed to 700 μM total ammonia still formed stable hierarchies but displayed lower levels of aggression in comparison to control hierarchies, a difference which attenuated in period 2. As well, 700 μM total ammonia hierarchies generally displayed lowered growth, lower condition factor increase and lower oxygen consumption during the first half of the experiment (period 1). These results were attributed to high ammonia's negative effect on swimming performance and appetite. However, during period 2, the fish exposed to 700 μM total ammonia displayed similar physiological parameters and feeding to those observed in control hierarchies, tough plasma cortisol levels were depressed in social ranks 1, 2, and 3. Overall, these results suggest that rainbow trout have the capability to alter both their behaviour and their physiology so as to acclimate to chronically elevated ammonia.

1. Introduction

Ammonia (the sum of NH_3 and NH_4^+) enters water systems through both natural and anthropogenic sources. Natural mechanisms include the production of nitrogenous waste from metabolic processes by plants, animals and microorganisms as well as atmospheric events such lightning. These processes, however, do not add significant amounts of nitrogen into waterways relative to those arising from human activities. Anthropogenic processes such as agriculture and industry place a large amount of nitrogen into both fresh- and salt-water ecosystems through run-off and sewage, respectfully. As such, ammonia has become an important aquatic pollutant affecting all aquatic life (Eddy 2006).

Total ammonia in water exists in two forms, NH_3 and NH_4^+. NH_3 is a gas and is protonated to the ionic form NH_4^+. Most cell membranes are permeable to NH_3 and thus ammonia gas is seen as the most important factor for ammonia toxicity. Various environmental factors such as pH, temperature, pressure and ionic concentration affect the dissociation of NH_3 to NH_4^+, with pH being the most prominent. For example, increasing the pH by a one unit causes a 10 fold increase in NH_3 (USEPA 1999).

Ambient water quality guidelines for the protection of freshwater aquatic life for ammonia toxicity have been developed in the United States and Canada. The USEPA (1999) has set an acute (24-h) total ammonia concentration of 1330 µM and a chronic value (96-h) of 330 µM at pH 7. These correspond to NH_3 concentrations of 3.4 and 0.75 µM, respectively. The Canadian Council of Ministers of the Environment (2000) has

reported a similar chronic value of 450 µM total ammonia for pH 7 (10°C), corresponding to an NH_3 concentration of 1.03 µM. The present study was conducted at total ammonia concentrations of 700, 1200 and 1550 µM (2.97, 5.10, 6.37 µM NH_3, respectively) at pH 7.2 (12°C). These concentrations are higher than chronic guidelines and were chosen to investigate the possible mechanisms by which such high external ammonia could affect the structure of social hierarchies and the physiology of individuals within them.

During exposure to high ammonia levels, some ammonia enters freshwater fish through their gills down the concentration gradient via diffusion (Wright et al., 2007). This increase of plasma ammonia causes stress to occur in the fish, resulting in increases in plasma cortisol concentrations and reductions in swimming performance (Shingles et al., 2001, Wicks et al., 2002c; Tudorache et al., 2008), growth (Foss et al., 2003), appetite (Ortega et al., 2005), and impaired ion regulation (Twitchen and Eddy, 1993; Wilson et al., 1994). However, high ammonia's effects on the formation of dominance hierarchies and the consequences of the ammonia burden on the physiology of individuals in a hierarchy have not been investigated.

Rainbow trout (*Oncorhynchus mykiss*), like most fish species, form social hierarchies when in groups. These hierarchies are the result of intense competition for limited resources such as food, shelter or mates. Normally, the fish that monopolizes the resources is known as the dominant individual in the group. The 'losing' fish is known as the subordinate.

Dominant and subordinate individuals have different physiological profiles from each other with dominant individuals displaying higher growth rates, higher food consumption and having access to mates (Pottinger and Pickering, 1992; McCarthy et al., 1992). Subordinates exhibit physical damage, lowered immunity, and slower growth rates (Abbot and Dill, 1985; Peters et al., 1988; McCarthy et al., 1992).

Stable hierarchies (in which social status does not change) are beneficial to both subordinate and dominant individuals by reducing aggressive behaviour compared to unstable hierarchies (Gurney and Nisbet, 1979). Environmental perturbations have the potential to alter social interactions within a group, leading to the collapse of hierarchies. Two such perturbations that are common in nature are reductions in water depth (resulting from droughts) and hypoxia conditions. Sloman et al. (2001) simulated drought conditions in the laboratory and showed that this led to the breakdown of brown trout hierarchies. Simulated drought conditions caused a similar result to occur in three-spined sticklebacks (Sneddon et al., 2005). In a different study, three-spine sticklebacks exposed to hypoxic conditions (20% oxygen) had less stable hierarchies with regards to rank position (Sneddon and Yerbury, 2004). These studies suggest that as the environment changes, so do the energetics dictating dominant and subordinate behaviour, resulting in the collapse of the hierarchy.

Aquatic toxicants, such as metals, can alter social behaviour which then leads to disruptive effects on dominance hierarchies in fish. Cadmium is one such metal. Sloman et al. (2003) showed that a reduction in aggressive behaviour occurred in rainbow trout

after just one day of cadmium exposure. However, cadmium did not cause degradation of dyad rainbow trout hierarchies – actually, hierarchies were established more quickly during cadmium exposure as compared to control treatments. Cadmium accumulation on the olfactory apparatus, reducing recognition between fish, is the likely reason for the observed difference in social behaviour (Scott et al., 2003). However, hierarchical degradation did not occur in rainbow trout exposed to copper (Sloman et al., 2001). Of interest, subordinate individuals did accumulate more copper in both the gill and liver compared to dominant fish. This suggests that social status resulting from a hierarchy can influence an individual's physiological response to a pollutant.

In Chapter 2, it was found that in tanks containing four rainbow trout, stable social hierarchies formed within the first 5 days of the 10 day experiment. Two physiologically different types of fish were created in these hierarchies: dominant and subordinate individuals. In these hierarchies formed in ammonia-free water, one fish would become the dominant fish while the other three fish became subordinate individuals with similar physiology. Dominant fish (social status rank 1) displayed higher growth, condition factor, food consumption, and relative protein utilization in metabolism combined with lower oxygen consumption compared to subordinate fish (social ranks 2, 3, and 4). It was also reported that individual aggressive acts correlated strongly with physiological parameters, creating a continuum of physiology and aggressive acts.

The present study investigated whether sublethal concentrations of water-borne ammonia would prevent the formation of a hierarchy in groups of four rainbow trout

(*Oncorhynchus mykiss)*, or alter its structure. Several levels of total ammonia were tested so as to assess concentration-dependency. Specifically, I hypothesized that the lowest effective ammonia concentration (found to be 700 µM total ammonia) would severely reduce appetite and aggression in the most dominant fish, resulting in healthier subordinate individuals compared to subordinates in hierarchies from ammonia-free water. If this were the case, then it would also be logical to hypothesize that the physiological profile of the ammonia-exposed hierarchies would be different than control hierarchies. Therefore, as part of this investigation, the physiological status of individuals in hierarchies exposed to 700 µM total ammonia (pH 7.2) were also recorded. As in Chapter 2, a new, non-invasive methodology was used to establish and then record physiological parameters in dominance hierarchies. The possible stress associated with this technique during the recording period was also evaluated.

2. Methods and Materials

2.1. Experimental animals, holding conditions and hierarchy preparation

Rainbow trout were the same size, obtained from the same location and held in exactly the fashion as stated in Chapter 2. The methods for creating, establishing and feeding social hierarchies were also exactly the same as stated in Chapter 2.

2.2 Experimental hierarchies

Seven control hierarchies were supplied with dechlorinated Hamilton tap water (water quality described in Chapter 2) for 11 days. Total ammonia concentration for inflow water was undetectable, while total ammonia concentration inside the tank ranged from undetectable to 6 µM total ammonia.

Three different ammonia concentrations (700, 1200 and 1500 µM) were created by adding analytical grade ammonium bicarbonate (NH_4HCO_3, Sigma-Aldrich, St. Louis, Missouri), dispensed through drip bottles, to hierarchical groups 12 hours before first feeding. These groups were set up in the exact same way as the control hierarchies. Two hierarchies were exposed to 1200 µM total ammonia, and two groups to 1500 µM total ammonia for 5 days. Six hierarchies were exposed to 700 µM total ammonia hierarchies for 11 days. Total ammonia concentrations were verified daily using a modified Verdouw et al. (1978) procedure with concentrations varying by no more than ±10 µM total ammonia from the nominal concentration for each concentration. Ammonium bicarbonate (NH_4HCO_3) was chosen as it did not appreciably alter the water pH, which fluctuated

between 7.2 and 7.4 (checked daily using a combination glass electrode (GK24O1C, Radiometer, Copenhagen, Denmark)) in the ammonia-exposed hierarchies.

To account for any effect that the high amount of bicarbonate in the ammonia exposed hierarchies (dissociation of NH_4HCO_3) might have on hierarchy structure or individual physiology (especially oxygen consumption rates), four hierarchies were established during exposure to 700 µM sodium bicarbonate, ($NaHCO_3$, Sigma-Aldrich, St. Louis, Missouri) using the exact same protocol as outlined for ammonia exposures, lasting for 11 days.

2.3. Behavioural measurements

Aggressive acts were measured during each morning feeding (15 minutes each feeding, 8 total feedings) using a video camera (Sanyo VPC-WH1, Osaka, Japan) set up on scaffolding surrounding the tanks. Each chase, approach and nip was given a point of 1, allowing each fish to have an aggressive score from which a social hierarchy can be established. For a detailed explanation on behavioural measurements, refer to Chapter 2.

2.4. Physiological measurements

Physiological parameters were recorded in starved fish (24 hours since last meal) by trapping and confining individual fish in a 'dummy' feeding apparatus on days 5 and 10, as described in Chapter 2. From here, fish were held for a total of 6 hours, with oxygen consumption rate measured within the first hour, and ammonia excretion rate

measurement requiring 6 total hours of confinement. Fish where then anesthetized in neutralized MS-222 (0.08 g L^{-1}), weighed (0.01 g) fork length (0.1 cm) measured and returned to their respective tanks for overnight recovery. Individual food consumption was measured on day 11 of the experiment, using the methodology of McCarthy et al. (1992). A thorough description can be found in Chapter 2.

2.5. Plasma cortisol collection

Six additional dominance hierarchies (in excess to the control, NH_4HCO_3, and $NaHCO_3$ hierarchies) were established in control water and held in exactly the same fashion as control hierarchies. These hierarchies were used to collect control plasma cortisol. Timeline, feeding and physiological measurements occurred as exactly as described above. On day 11, fish from these additional control hierarchies, as well as fish from the six hierarchies exposed to 700 μM total ammonia were euthanized using a concentrated dose of MS-222 (5 g L^{-1}, neutralized) so as to minimize stress. Blood was collected via tail severance and centrifuged in order to obtain plasma. Blood plasma was stored at -20^{0}C until assayed with Cayman Chemical EIA Kit (Ann Arbor, Michigan).

2.6. Assessment of stress associated with confinement for respirometry

To determine whether using the 'dummy' feeding container to confine individual fish for respirometry measurements caused the occurrence of stress, four hierarchies were established as outlined above (refer to Chapter 2). One fish per tank was removed each

day by using the 'dummy' feeding container and the fish was left inside the 'dummy' container for 0, 0.5, 1, or 3 hours with aeration. These fish were then terminally sampled (MS-222, 5 g L^{-1}, neutralized) to obtain blood plasma. Only one fish was removed per tank per day to minimize any stress to the remaining fish. A separate group of fish that had an established social hierarchy were sampled to obtain control cortisol values. In order to sample control hierarchical fish, the tank water was slowly lowered and a concentrated dose of neutralized MS-222 (2 g/L) was added to rapidly anesthetise the fish. Blood samples were taken via caudal vein puncture, with all fish being air-exposed for less than 2 min with no mortality resulting from procedure. Cortisol was assayed with Cayman Chemical EIA Kit (Ann Arbor, Michigan).

2.7. Calculations

The same calculations for growth, Fulton's condition factor, oxygen consumption and ammonia excretion were performed as in Chapter 2.

2.8. Statistical analyses

SigmaStat 3.5 (Systat Software, Inc. 2006) and Statistica 7.0 (StatSoft Inc. 2004) software were both used to perform statistical analyses. One-way ANOVA followed by the Fisher's Least Significant Difference (LSD) post-hoc test were used to test for differences in aggressive acts per day among control, 700, 1200 and 1500 µM total ammonia-exposed hierarchies. Kruskal-Wallis one-way ANOVA analyses were

conducted to test for differences in feeding and aggressive acts per day between control and 700 µM total ammonia-exposed hierarchies. Two-way ANOVA and the Fisher's LSD post-hoc test were performed to test for differences in growth, percent change in condition factor, oxygen consumption, ammonia excretion, and plasma cortisol between the control and 700 µM total ammonia hierarchies. Oxygen consumption, ammonia excretion and plasma cortisol data were log transformed to fit normality. Differences in aggressive acts per day per hierarchy between control and 700 µM total ammonia exposed hierarchies were tested by t-test. A paired t-test with Bonferroni correction was used to analyse aggressive acts per day within control and 700 µM total ammonia treatments.

3. Results

3.1. Behavioural measurements

3.1.1. Aggressive acts performed by each exposure and hierarchy formation

Control hierarchies (with non-detectable total ammonia concentrations) exhibited significantly higher aggressive acts per day than any of the other ammonia-exposed treatments. Controls displayed 20.0 aggressive acts per day compared to 8.7 and 5.0 aggressive acts per day for 700 and 1200 µM total ammonia groups, respectively (Fig. 1). There was no significant difference between aggressive acts per day performed between 700 and 1200 µM total ammonia groups. 1500 µM total ammonia treatments did not display any aggressive acts.

Social hierarchies were formed in both control and 700 µM total ammonia treatments. However, due to the very low occurrence of aggressive acts, dominance hierarchies could not be determined in 1200 and 1500 µM total ammonia treatments. Therefore, physiological parameters were not recorded in these groups. 700 µM total ammonia hierarchies will be referred to as 'Ammonia hierarchies' for the remainder of the chapter.

The four hierarchies treated with 700 µM $NaHCO_3$ (data not shown) displayed very similar patterns to the Control hierarchies,(e.g. comparable aggression, O_2 consumption patterns, growth rate patterns etc.) which were different from those treated with 700 µM NH_4HCO_3 . Thus the effects seen in the Ammonia hierarchies were attributable to elevated ammonia, and not to elevated bicarbonate.

3.1.2. Aggressive acts per day in control and 700 μM total ammonia hierarchies

Average aggressive acts per day was significantly higher in Control hierarchies (t-test, p=<0.001) than in Ammonia hierarchies (20.1 and 8.7 aggressive acts per day respectively). Control hierarchies tended to display higher aggressive acts per day than Ammonia hierarchies through until day 8 (Fig. 2). On day 6 and 7, control hierarchies displayed significantly higher aggressive acts than 700 μM total ammonia hierarchies (23.0 and 24.2 for control, respectively and 9.2 and 9.3 for Ammonia, respectively). There were no significant differences at other individual days.

Within Control or Ammonia hierarchies, each day had a similar aggressive acts frequency throughout the experiment, with no days being significantly different (Fig. 2). The apparent decline in aggression frequency on days 8 and 9 in the Controls was not significant. There was only one observable switch in social status from period 1 to period 2, occurring in the Control hierarchies where one fish switched from rank 1 to rank 2 and vice versa. This did not occur in the Ammonia hierarchies.

3.1.3. Aggressive acts per day based on social status

During period 1, there was a significant difference among ranks regardless of treatment hierarchies, with Control hierarchies displaying more aggressive acts per day (p=<0.001). Control rank 1 fish had significantly higher aggressive acts per day than all other fish (14.3) (Fig. 3). Ammonia rank 1 and Control rank 2 fish had similar values, 4.8

and 7.0 aggressive acts per day, respectively, which was significantly higher than Ammonia ranks 3 and 4 (0.4 and 0.3 aggressive acts per day) as well as Control ranks 3 and 4 individuals (0.9 and 0.4 aggressive per day). Ammonia rank 2 individuals exhibited 2.4 aggressive acts per day which did not significantly differ from Control rank 2, 3 and 4 as well as Ammonia rank 1, 2, 3 and 4.

In period 2, there was a significant difference among ranks in Control and Ammonia hierarchies ($p=<0.001$), with no difference being observed within rank between Control and Ammonia hierarchies (Fig. 3). Control and Ammonia rank 1 fish displayed similar aggressive acts per day (11.4 and 9.7, respectively) during period 2, which did not significantly differ from each other. Control rank 2 and Ammonia rank 2 also did not significantly differ in aggressive acts per day, 4.5 and 3.6, respectively. Control rank 3 and 4 exhibited similar aggressive acts per day as Ammonia rank 3 and 4 (2.0 and 0.4 for Control, respectively, and 0.7 and 0.0 for Ammonia, respectively). These values did not significantly differ from each other.

3.2. Physiological parameters for each social status in control and 700 μM total ammonia

3.2.1. Specific growth rate

During period 1, there was a significant overall difference between Control and Ammonia hierarchies ($p=0.007$), as well as a significant difference among ranks regardless of exposure ($p=<0.001$). Control rank 1 fish had a growth rate of 2.6%, which

was not significantly different from Ammonia rank 1 fish that had a 1.1% growth rate (Fig. 4). Control and Ammonia rank 2 fish did not significantly differ in growth rate (0.5 and 0.7%, respectively) and were not significantly different than Ammonia rank 1. Growth rates among Control rank 3 and 4 and Ammonia rank 3 fish did not differ significantly (-0.3. 0.01 and -1.3%, respectively). Ammonia rank 4 individuals had the lowest specific growth rate (-2.7%) and were significantly different compared to other social ranks.

During period 2, there was no longer an overall significant difference between Control and Ammonia hierarchies ($p=0.701$), however, a significant difference among ranks persisted regardless of the exposure ($p=<0.001$) (Fig. 4). Control rank 1 fish displayed the highest growth rate (2.3%) compared to the other fish, however, this growth rate was not significantly different than Ammonia rank 1 (1.7%). Growth rate of Ammonia rank 1 fish was not significantly different than Control and Ammonia rank 2 fish (0.8 and 0.7%, respectively). Both Control and Ammonia rank 3 fish exhibited similar low growth rates (0.4 and 0.6%, respectively). Control and Ammonia rank 4 fish also displayed growth rates which were not significantly different (-0.6 and -0.7%, respectively). Ammonia ranks 1, 2 and 3 individuals had similar growth rates to Control rank 2 fish. Control ranks 2, 3 and 4 fish and Ammonia ranks 2 and 3 did not significantly differ between each other, with Ammonia rank 4 individuals displaying the lowest growth rate in period 2.

3.2.2. Percent change in condition factor

In period 1, there was a significant overall difference in percent change in condition factor between Control and Ammonia hierarchies (p=<0.036) as well as a significant difference among the ranks of both Control and Ammonia hierarchies (p=0.003) (Fig. 5). Control rank 1 and Ammonia rank 1 fish did not significantly differ in their percent change in condition factor (12.6 and 4.6%, respectively). Actually, Control ranks 2, 3, 4 and Ammonia ranks 1, 2 and 3 all displayed non-significantly different percent change in condition factor (0.9, 0.5, 1.0% for control ranks, respectively, and 4.6, 3.5 and -5.1% for Ammonia ranks, respectively). Ammonia rank 4 individuals had the lowest percent change in condition factor, -9.3%, but this was not significantly different than Ammonia rank 3 fish.

During period 2, there was no longer a significant overall difference between control and Ammonia hierarchies (p=0.693) (Fig. 5). However, there was a significant difference among ranks regardless of the exposure (p=<0.001). Control ranks 1 and 2 and Ammonia ranks 1 and 2 displayed similar percent change in condition factor (8.6, 2.4, 6.8, and 4.0% for Control and Ammonia, respectively), which did not significantly differ from each other. Control ranks 3 and 4 (2.0 and -1.4%) and Ammonia ranks 3 and 4 (2.1 and -4.1% change in condition factor) did not significantly differ from each other. Control ranks 2 and 3 were not significantly different than Ammonia ranks 2 and 3.

3.2.3. Percent feeding

Feeding was quantified only on day 11, the final day of the experiment. Ammonia exposure did not affect the amount of food consumed on this day, as the entire meal was consumed in both treatments. But among ranks within Control and Ammonia hierarchies, there was a significant difference in the percentage food consumed ($p=<0.001$). Control rank 1 consumed 66.9% of the meal, while Ammonia rank 1 consumed 84.5% (Fig. 6). There was no significant difference between these two values. Control rank 2 and Ammonia rank 2 fish had similar consumption levels, consuming 23.2% and 17.8%, respectively. Rank 3 fish from both Control and Ammonia hierarchies consumed non-significantly different amounts of food, 5.7% and 10.7%. Lowest food consumption was observed in Ammonia rank 4 fish, but this was not significantly different than Control rank 4 (1.0% compared to 4.2%, respectively).

3.2.4. Oxygen consumption

Overall, higher oxygen consumption rates were observed in the Control relative to Ammonia hierarchies, during period 1, a difference which just escaped significance ($p=0.056$) (Fig. 7). There was also not a significant overall influence of social rank ($p=0.798$).Control rank 1 fish had an oxygen consumption rate of 13.0 $\mu mol\text{-}g^{-1}h^{-1}$ which was the lowest observed (Fig. 7), but was not significantly different than Control ranks 2, 3 and 4 (20.3, 25.5, 22.4 $\mu mol\text{-}g^{-1}h^{-1}$). All Ammonia ranked fish tended to have the similar oxygen consumption rates: 15.3, 10.7, 13.4 and 14.7 $\mu mol\text{-}g^{-1}h^{-1}$ for rank 1, 2, 3

and 4 respectively. The only significant difference between ranks was observed between Control rank 3 and Ammonia rank 2 individuals.

During period 2, there was no significant difference between control and Ammonia hierarchies with regards to oxygen consumption (p=0.209), as well as no significant difference among ranks regardless of the exposure (p=0.268). All ranks of fish from both Control and Ammonia hierarchies displayed similar oxygen consumption rates (Control hierarchies: 13.7, 24.9, 22.4 and 24.0 $\mu mol\text{-}g^{-1}h^{-1}$ for ranks 1, 2, 3 and 4, respectively; Ammonia hierarchies: 13.6, 18.7, 15.5, 19.5 $\mu mol\text{-}g^{-1}h^{-1}$ for ranks 1, 2, 3 and 4, respectively).

3.2.5. Ammonia excretion

In period 1, there was no significant overall difference in ammonia excretion between Control and Ammonia hierarchies (p=0.160) (Fig. 8). There was also no significant difference among ranks regardless of exposure (p=0.671). Control ranks 1, 2, 3 and 4 individuals all excreted ammonia at similar rates (1.4, 0.9, 0.9, and 0.7 $\mu mol\text{-}Ng^{-1}$ h^{-1} , respectively) , values which were not significantly different than those of Ammonia ranks 1, 2, 3 and 4 fish (2.6, 1.2, 2.0, 3.3 $\mu mol\text{-}Ng^{-1}$ h^{-1}, respectively).

During period 2, there was again no significant difference between Control and Ammonia hierarchies in terms of ammonia excretion (p=0.398), as well as among ranks within control or Ammonia hierarchies (p=0.647) (Fig. 8). Control rank 1 and Ammonia rank 1 fish displayed similar ammonia excretion rates, 1.5 and 1.1 $\mu mol\text{-}Ng^{-1}$ h^{-1},

respectively. These excretion rates were not significantly different than those of Control ranks 2, 3 and 4 (0.8, 1.1, 0.7 µmol-Ng^{-1} h^{-1} , respectively) or Ammonia ranks 2, 3, and 4 (1.5, 2.0, 2.1 µmol-Ng^{-1} h^{-1} , respectively). The only significant difference was observed between Control rank 4 (0.7 µmol-Ng^{-1} h^{-1}) and Ammonia rank 4 (2.1 µmol-Ng^{-1} h^{-1}) individuals.

3.2.6. Plasma cortisol

Overall, there was a significant difference in plasma cortisol concentrations between Control and Ammonia hierarchies, with Ammonia hierarchies having lower plasma cortisol concentration compared to Control hierarchies ($p=<0.001$) (Fig. 9). But there was no a significant difference among ranks in Control and Ammonia hierarchies ($p=0.287$). Control rank 1 and Ammonia rank 1 fish had significantly different plasma cortisol levels, with Control rank 1 having 59.5 and Ammonia rank 1 having 17.1 ng/ml plasma cortisol. Rank 2 values between Control and Ammonia hierarchies were significantly different, 88.2 and 18.6 ng/ml plasma cortisol, respectively. Control rank 3 fish had significantly higher plasma cortisol (130.0 ng/ml) compared to Ammonia rank 3 individuals (35.6 ng/ml). Control rank 4 and Ammonia rank 4 did not differ significantly (116.1 compared to 87.8 ng/ml, respectively). Control ranks 1, 2, 3 and 4 did not differ significantly from each other.

3.3. Assessment of stress associated with confinement in the respirometer

The method of confining fish in a 'dummy' feeding container for respirometry appears to cause a cortisol response in individual fish (Fig. 10). The 0-h cortisol concentration of 20 ng/ml, measured immediately after transfer to the respirometer, was not significantly different for the control value of 15 ng/ml measured in the fish sampled directly in a hierarchy. However trout held inside the darkened container for 0.5h had levels of 55 ng/ml, and by 1 h, corresponding to the period used for oxygen consumption measurements, plasma cortisol concentrations had reached 75 ng/ml, which was significantly higher than both the control and 0-h levels. After 3h of holding inside the container, cortisol averaged 97 ng/ml, which was not significantly greater than the 1-h level. Social status of the fish had no effect on the increased cortisol response.

4. Discussion:

This is the first study, to the author's knowledge, to investigate the effect of high external ammonia on social hierarchies in rainbow trout. Dominance hierarchies formed in both Control and 700 µM total ammonia groups but not in the 1200 or 1500 µM total ammonia treatments, with more aggression being observed in the Control hierarchies (Figs. 1, 2). The difference in aggression was especially noticeable during period 1, with this trend reversing during period 2 (Fig. 3). In the 700 µM total ammonia hierarchies, lower specific growth rate and lower percent change in condition factor were recorded compared to Control hierarchies, a difference which was especially clear during period 1 (Figs. 4, 5). During period 2, these trends 'rebounded' and the Ammonia hierarchies exhibited similar patterns to those of the Control hierarchies. Ammonia hierarchies had lower plasma cortisol concentrations compared to Control hierarchies (Fig. 9), while feeding percentage was similar between these two exposures (Fig. 6). Generally speaking, Ammonia hierarchies had lower oxygen consumption (Fig. 7) while having higher ammonia excretion rates than Control hierarchies in both period 1 and period 2 (Fig. 8). Taken together, these results suggest that high external ammonia caused a reduction in aggression, which in turn could explained lowered oxygen consumption rates. At the two highest total ammonia concentrations (1200 and 1500 µM total ammonia), aggression was reduced so severely that a social hierarchy could not be determined. It also appears that fish exposed to 700 µM total ammonia have developed some detoxification or

acclimation mechanisms during the course of the experiment, as many physiological parameters had returned to control levels during period 2.

The reduction in aggression observed in the Ammonia hierarchies (period 1) may be attributed to reduced swimming performance caused by increased plasma ammonia levels. Past research has correlated reduced swimming performance with increased plasma ammonia concentrations in brown trout, rainbow trout, and coho salmon (Beaumont et al., 1995; Shingles et al., 2001; Wicks et al., 2002). High total ammonia levels in the water leads to elevated plasma ammonia in fish as NH_3 passively diffuses down the concentration gradient across the gills. From here, ammonia also accumulates in the muscle tissue, which can disrupt both anaerobic and aerobic metabolism (Beaumont et al., 2000a). This would lead to reduced aggression as muscle metabolism is compromised. Recently, Tudorache and De Boeck (2008) demonstrated that elevated water ammonia levels (60 µM total ammonia or 1.2 µM NH_3, pH 7.8) reduced fast starts in brown trout, leading to reduced agonistic behaviour in dominant individuals. In the current study at 700 µM total ammonia at (2.7 µM NH_3, pH 7.2) reduced aggression was observed during period 1 in the higher social status fish, whereas lower social status fish of Control and Ammonia hierarchies displayed similar levels of aggression (Fig. 3).

There are several possible mechanisms that cause a reduction in swimming performance as a result of increased plasma ammonia. Firstly, accumulated ammonia can disrupt biochemical processes. Ammonia can have a stimulatory effect on phosphofructokinase which can increase the rate of glycolytic flux, reducing glycogen

reserves, thereby potentially affecting anaerobic work potentially (Beaumont et al., 2000; McKenzie et al., 2003). Ammonia can also limit aerobic metabolism by inhibiting various steps of the citric acid cycle (Lai and Cooper, 1991). Ammonia can also affect neurological function by substituting NH_4^+ for K^+ (Cooper and Plum, 1987). By a similar mechanism, it is believed that NH_4^+ can accumulate in muscle cells leading to muscle depolarization (Beaumont et al., 1995).

Other studies have also been reported reduced activity levels in fish exposed to high ammonia concentrations. Schools of Koi fish dived down and remained at the lower depth for an extended period of time at the beginning of exposure to elevated environmental ammonia (6.7 – 44.5 µM total ammonia or 0.07 – 0.50 µM NH_3, pH 7.4). (Israeli-Weinstein and Kimmel, 1998). These ammonia-exposed Koi also showed decreased activity, formed a tighter shoal and decreased food consumption. Tudorache et al. (2010) also showed a reduction in swimming performance in brook charr exposed to high total ammonia (43.1 – 57.5 µM total ammonia or 12.5 – 16.6 µM NH_3, pH 9.1) levels for four days.

However, it appears that during period 2, swimming performance was not reduced as aggression levels based on social status were no longer different between Control and Ammonia hierarchies (Fig. 3). This possibly suggests a detoxification mechanism or acclimation to the high ammonia occurred, allowing for aggression to return to Control levels. Certainly, similar patterns of "rebound" were seen in certain physiological parameters (discussed below). Nevertheless, total levels of aggression on day 6 and day 7

were still significantly higher in Control hierarchies (Fig. 2), so this conclusion should be treated with caution. More studies are needed to clarify this matter.

Another factor to consider is duration of experiment. In the aforementioned studies reporting reduced swimming performance due to increased plasma ammonia levels, four days of exposure to toxicant was the longest duration. This leaves the possibility open that there might not be a reduction in swimming performance due to high plasma ammonia after 4 days of exposure, supporting that either detoxification or acclimation to the high ammonia environment is occurring during period 2 (Fig. 3).

A tendency for reduced oxygen consumption was observed in the Ammonia hierarchies (Fig. 7), particularly in period 1. This can be partially explained by the reduced level of aggression that took place in the Ammonia hierarchies. Another possible explanation could be the reduced metabolic demand in fish exposed to high water-born total ammonia (as discussed above). Chronic exposure of walleye (*Sander vitreus)* to moderate levels of ammonia (300 µM total ammonia or 4.7 µM NH_3, pH 7.6), resulted in a significant decrease in oxygen consumption compared to unexposed fish (Madison et al., 2009). The authors attributed this result to decreased muscle metabolic demand and lower plasma cortisol levels. Elevated plasma cortisol has been shown to increase oxygen consumption (Morgan and Iwama, 1996; Sloman et al., 2000; De Boeck et al., 2001). However in the current study, plasma cortisol measurements were not taken on day 5 so cortisol's short-term influence cannot be determined. During period 2, Ammonia

hierarchies displayed oxygen consumption rates similar to control hierarchies (discussed below).

Lower growth rate and lower percent change in condition factor were observed in the Ammonia hierarchies (Figs. 4, 5) during period 1. Past research has demonstrated that elevated total ammonia causes lower growth and a reduction in food consumption (Person-Le Ruyet et al., 1997; Foss et al., 2003; Wicks and Randall, 2002a; Ortega et al., 2005). Indeed, by anecdotal observation it is believed that Ammonia hierarchies during period 1 exhibited reduced appetite and food consumption ultimately resulting in lowered growth and lower percent change in condition factor. Feeding consumption was recorded only on day 11 so this conclusion cannot be verified.

However, the feeding measurement taken on day 11 indicated that Control and Ammonia hierarchies displayed similar food consumption at this time (Fig. 6). During period 2 (ending on day 10), growth (Fig. 4) and change in condition factor (Fig. 5) were also similar between control and Ammonia hierarchies. This supports the previously stated idea that appetite suppression occurred during period 1, resulting in reduced growth and condition factor. It appears that the fish in Ammonia hierarchies have modified their biochemistry or physiology in order to handle the elevated ammonia environment, so as to become similar to Control fish during period 2.

Alternately, if it is assumed that percent feeding for each social status was similar in the preceding days to what was recorded on day 11, then this would suggest that 700 μM total ammonia affected the food conversion efficiency, since growth and condition

factor were lower compared to control hierarchies during period 1 but food consumption would be the same. However, this is unlikely as most available data suggests that exposure to high ammonia negatively affects food consumption, but not feeding efficiency (Person-Le Ruyet et al., 1997; Foss et al., 2003; Foss et al., 2004).

In a recent study, Foss et al. (2009) reported that a high pulse or high chronic ammonia concentration (360 or 680 µM total ammonia or 9.4 or 17.8 µM NH_3, respectively, pH= 8.04) caused a reduction in growth but not food consumption or food conversion efficiency in juvenile turbot. No reason was given for this observation, but it does suggest that elevated water-borne total ammonia concentrations may possibly affect growth negatively through an alternative mechanism.

It is interesting to note that ammonia excretion tended to be higher in the Ammonia hierarchies than control (Fig. 8), which has been previous shown to occur (Wood 2004) at low to moderately high total ammonia concentrations (70 and 225 µM or .0.8 and 2.86 µM NH_3 ,pH=7.6). It is not known why this occurs, but it appears that water-borne ammonia has a stimulatory effect on ammonia excretion. One possibility is that the Ammonia fish are utilizing protein more for aerobic metabolism than fish in the Control hierarchies. However, calculation of percent protein utilization from the individual MO_2 and M_{Amm} measurements, as in Chapter 2, revealed no significant differences between the treatments (data not shown).

Plasma cortisol was generally lower in the Ammonia-exposed hierarchies than in the Controls (Fig. 10). It has been shown previously that high ammonia does elicit a

cortisol response (Person-Le-Ruyet et al., 1998; Wicks and Randall, 2002B; Ortega et al., 2005) so the current finding may be somewhat unexpected. However, as discussed earlier, lower plasma cortisol has also been seen during chronic ammonia exposure in at least one previous study (Madison et al., 2009). Notably, lower aggression is observed in the Ammonia hierarchies which could result in lower plasma cortisol levels. Between dyads of rainbow and brown trout, the magnitude of the cortisol rise has been correlated with the strength of a hierarchy (Sloman et al., 2001b). Moreover, both Madison et al. (2009) and Ortega et al. (2005) report cortisol levels returning to control levels by the end of their ammonia exposure experiments (56 days and 4 days, respectively). This suggests that there might be a possible detoxification or acclimation mechanism occurring in fish chronically exposed to ammonia. However, this does not explain the low plasma concentrations observed in the Ammonia hierarchies. Recently, it was demonstrated that rainbow trout chronically exposed to cupper (30 µg Cu/L for 40 days) could not elicit a cortisol response to a second stressor compared to control fish (Tellis et al., 2011). Pickering and Pottinger (1987) also reported a similar finding in brown trout held in poor water quality conditions. It has been suggested that a reduced cortisol response might be an energy-saving mechanism, allowing fish to invest in other physiological processes such as growth and repair (Madison et al., 2009).

It appears that Ammonia hierarchies 'rebounded' in period 2, as many physiological parameters were no longer different from those observed in Control hierarchies. Fish in the Ammonia hierarchies may have altered their biochemical

processes during the experiment in order to handle the elevated ammonia concentrations. This 'rebound' has previously been shown in juvenile seabass *(Dicentrarchus labrax)* exposed to above 600 µM total ammonia at pH=8 (18.8 µM NH_3) (Lemarie et al., 2004). These scientists found that after 13 days (during which negative growth occurred) growth rate returned to control levels. They suggested that glutamine synthetase most likely reduced ammonia toxicity in seabass exposed to high ammonia levels by transforming glutamate to glutamine by adding a NH_4^+ molecule. Once fish have overcome the ammonia toxicity, then physiology returns what is seen in Control hierarchies.

4.1. Stress associated with confinement in the respirometer

Ideally, non-invasive methods for assessing the physiologies of fish in hierarchies should not cause stress to the animals, although this is rarely evaluated. One exception is the study of Brydges et al. (2009). These authors used a darkened scoop designed to hold water when capturing rainbow trout and compared it to a traditional net. The darkened scoop caused the same cortisol response as a net. In the present study, it was hoped that confining fish non-invasively inside a darkened container for respirometry would not cause stress, especially since the fish were captured without disturbance in the "dummy" feeding container. Nevertheless, a clear mobilization of cortisol was evident within 30-60 min and this persisted for at least 3h (Fig. 9). Whether this was due to isolation, confinement, continued darkness, or other factors is unclear. Nevertheless, interpretation

of the respirometry measurements (oxygen consumption, ammonia excretion) should take into account the possible confounding effects of elevated cortisol.

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Figure 1. Aggressive acts per day per group exposed to different concentrations of total ammonia. Control and 700 µM total ammonia hierarchies were exposed for 10 days, while 1200 µM total ammonia and 1500 µM total ammonia were exposure for 5 days. Values are means of hierarchical groups ± S.E.M. Different letters denote significant differences between exposure groups. (p=0.035, One-way ANOVA; post-hoc test Fisher LSD) (control N=7; 700 µM total ammonia N=6; 1200 µM total ammonia N=2; 1500 µM total ammonia N=2).

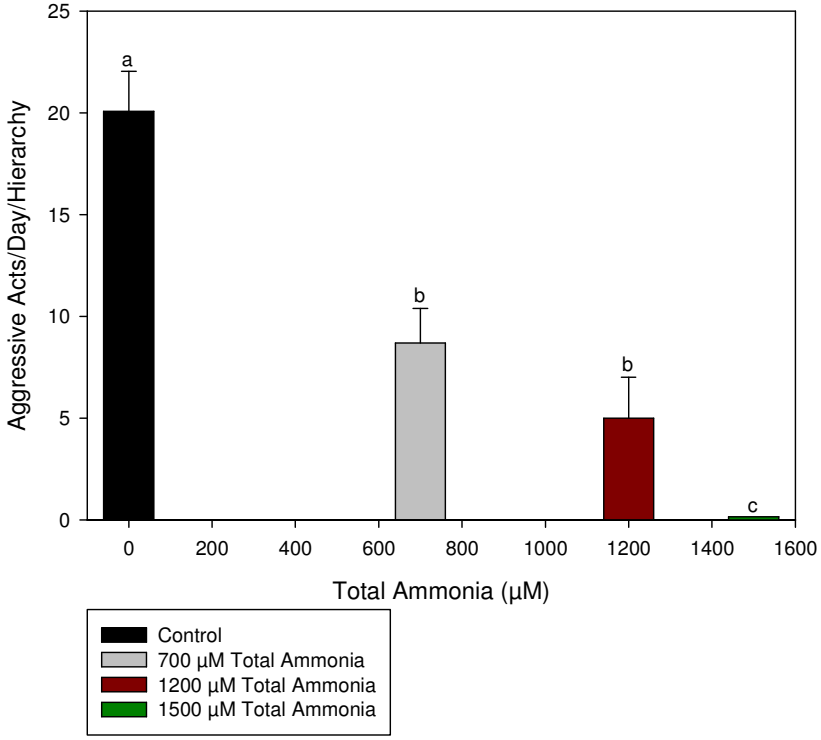
25
20
15
10
5
0
Aggressive Acts/Day/Hierarchy
a
b
b
c
0
200
400
600
800
1000
1200
1400
1600
Total Ammonia (µM)
Control
700 µM Total Ammonia
1200 µM Total Ammonia
1500 µM Total Ammonia

Figure 1.

Figure 2. Aggressive acts per day for control and 700 µM total ammonia hierarchies. Values are means of hierarchical groups ± S.E.M. Significant differences between aggressive acts per day for control and 700 µM total ammonia denoted by asterisk. (Control N=7; 700 µM total ammonia N=6).

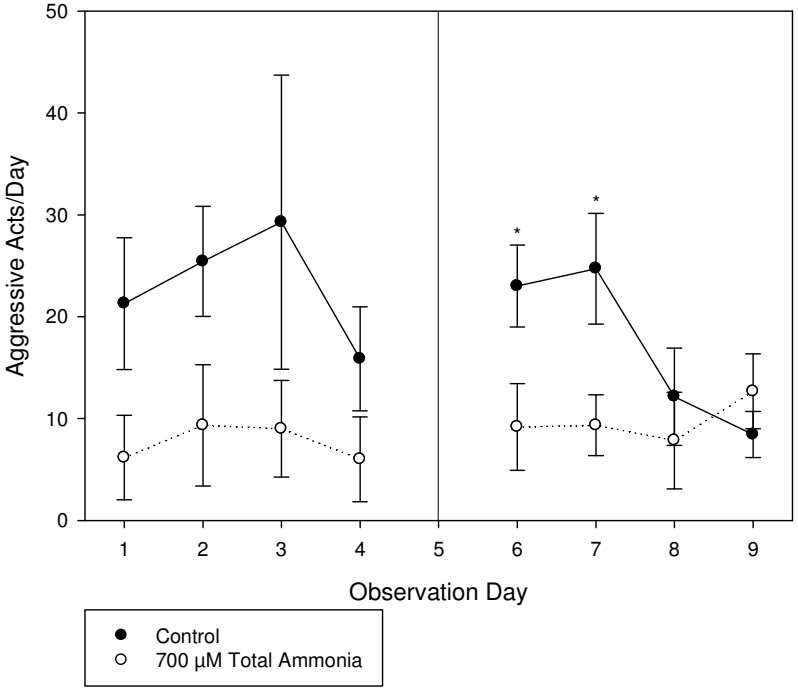
50
40
30
20
10
0
Aggressive Acts/Day
1
2
3
4
5
6
7
8
9
Observation Day
*
*
Control
700 µM Total Ammonia

Figure 2.

Figure 3. Aggressive acts per day based on social status for control and 700 µM total ammonia hierarchies during period 1 and period 2. Values are means ± S.E.M. Different letters denote significant differences between ranks. Period 1: a significant difference exists among ranks regardless of exposure (p=<0.001). Period 2: a significant difference exists between ranks in Control and 700 µM total ammonia hierarchies (p=<0.001) (Control N=7; 700 µM total ammonia N=6). (Kruskale-Wallis one-way ANOVA based on social status; post-hoc test Fisher LSD).

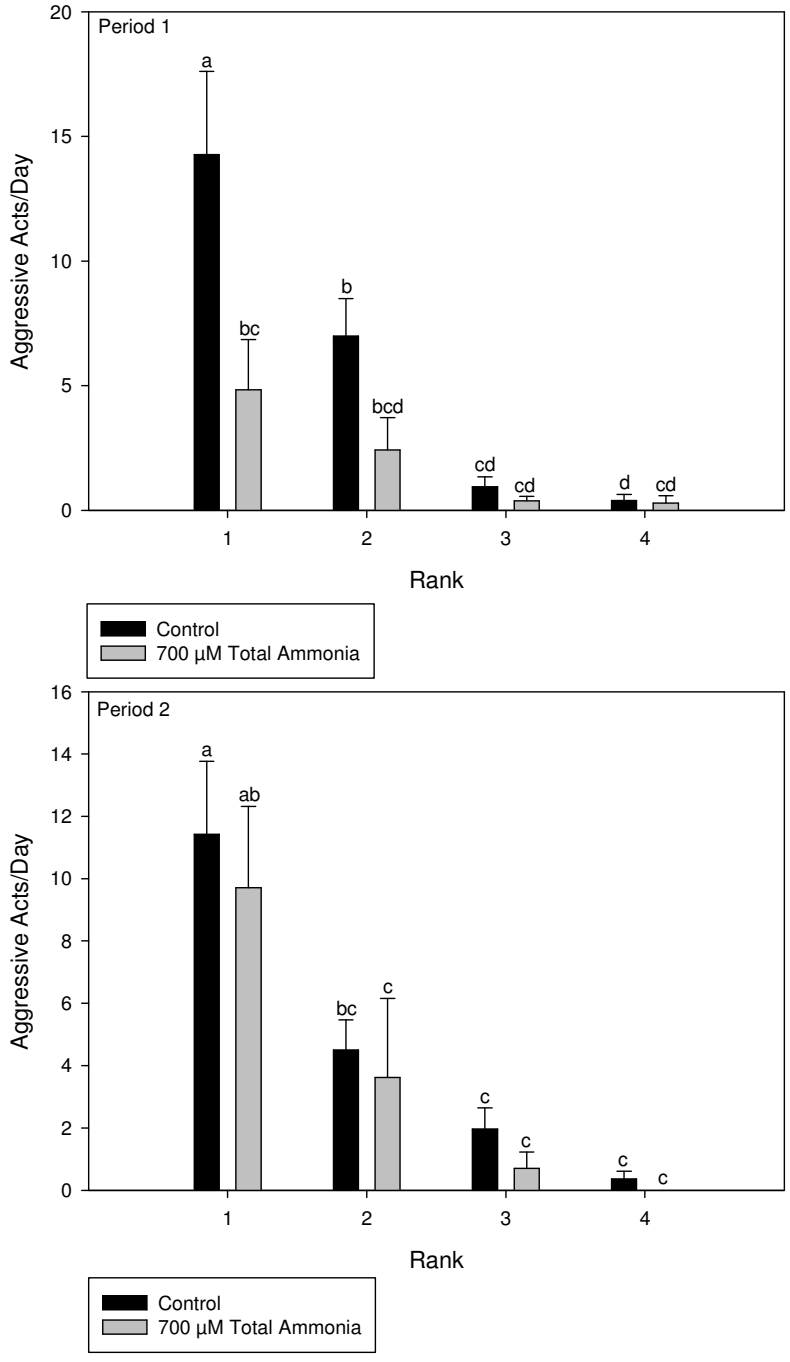
Period 1
Aggressive Acts/Day
Rank
a
bc
b
bcd
cd
cd
d
cd
Control
700 µM Total Ammonia
Period 2
Aggressive Acts/Day
Rank
a
ab
bc
c
c
c
c
c
Control
700 µM Total Ammonia

Figure 3.

Figure 4. Specific growth rate based on social status for control and 700 µM total ammonia hierarchies during period 1 and period 2. Values are means ± S.E.M. Different letters denote significant difference. Period 1: a significant difference exists between control and 700 µM total ammonia hierarchies (p=0.007). There is also a significant difference among ranks regardless of exposure (p=<0.001). Period 2: there is no significant difference between control and 700 µM total ammonia hierarchies (p=0.701). There is a significant among ranks regardless of exposure (p=<0.001) (Control N=7; 700 µM total ammonia N=6; Two-way ANOVA; post-hoc test Fisher LSD).

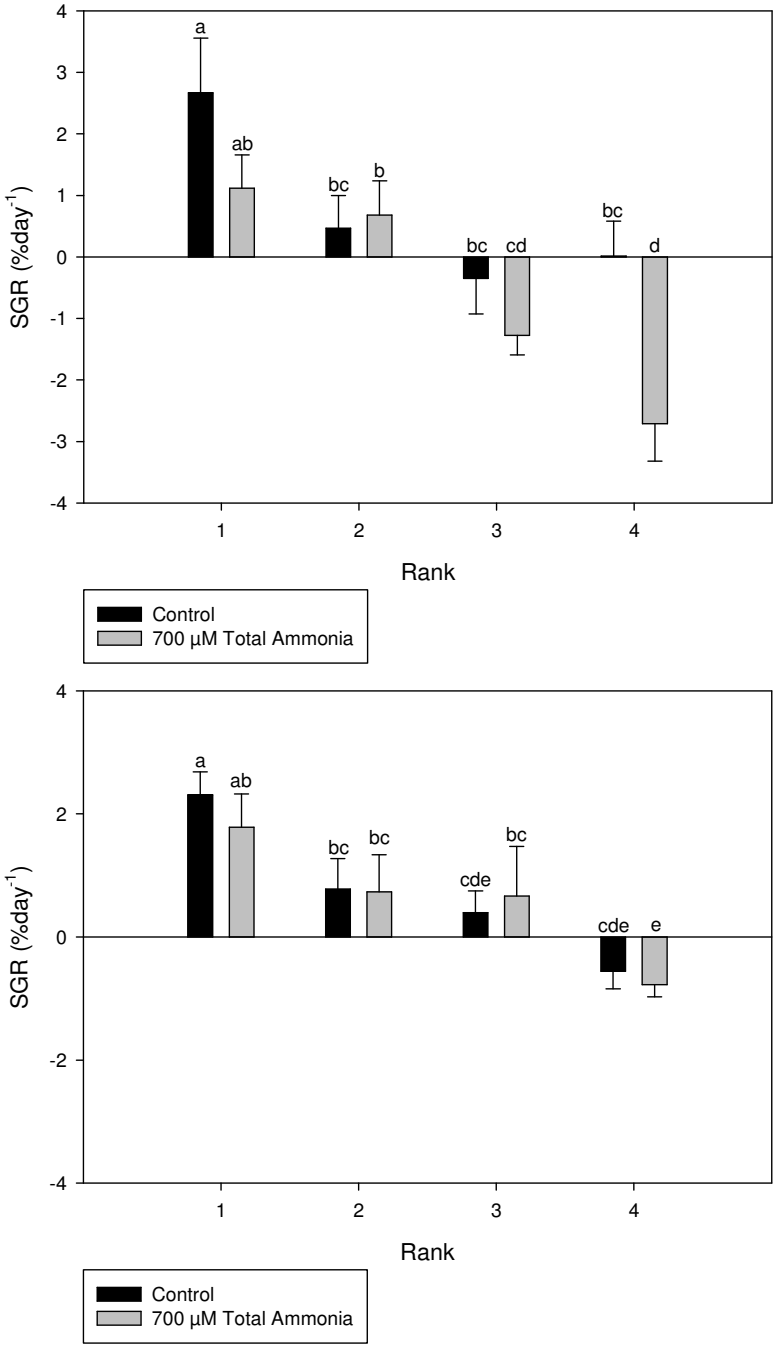
SGR (%day-1)
Rank
a
ab
bc
b
bc
cd
bc
d
Control
700 µM Total Ammonia
SGR (%day-1)
Rank
a
ab
bc
bc
cde
bc
cde
e
Control
700 µM Total Ammonia

Figure 4.

Figure 5. Percent change in condition factor based on social status for control and 700 µM total ammonia hierarchies during period 1 and period 2. Values are means ± S.E.M. Different letters denote significant difference. Period 1: significant difference exists between control and 700 µM total ammonia hierarchies (p=0.036). There is also a significant difference among ranks regardless of exposure (p=0.003). Period 2: there is no significant difference between control and 700 µM total ammonia hierarchies (p=0.693). There is a significant difference among ranks regardless of exposure (p=<0.001) (Control N=7; 700 µM total ammonia N=6; Two-way ANOVA; post-hoc test Fisher LSD).

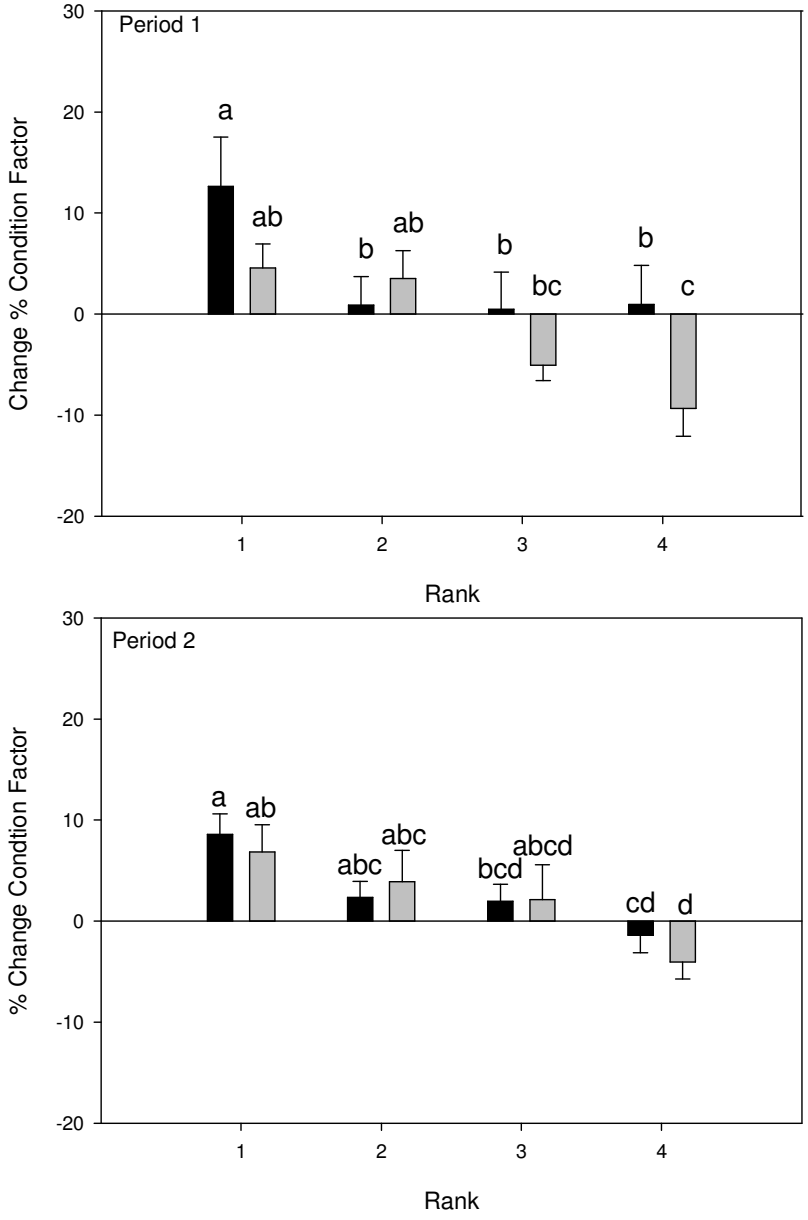
Period 1
Change % Condition Factor
30
20
10
0
-10
-20
a
ab
b
ab
b
bc
b
c
1
2
3
4
Rank
Period 2
% Change Condtion Factor
30
20
10
0
-10
-20
a
ab
abc
abc
bcd
abcd
cd
d
1
2
3
4
Rank

Figure 5.

Figure 6. Percent feeding for one meal (on day 11) based on social status for control and 700 µM total ammonia hierarchies. Values are means ± S.E.M. Different letters denote significant difference. There is a significant difference among ranks regardless of exposure (p=<0.001). (Control N=7; 700 µM total ammonia N=6; Two-way ANOVA; post-hoc test Fisher LSD).

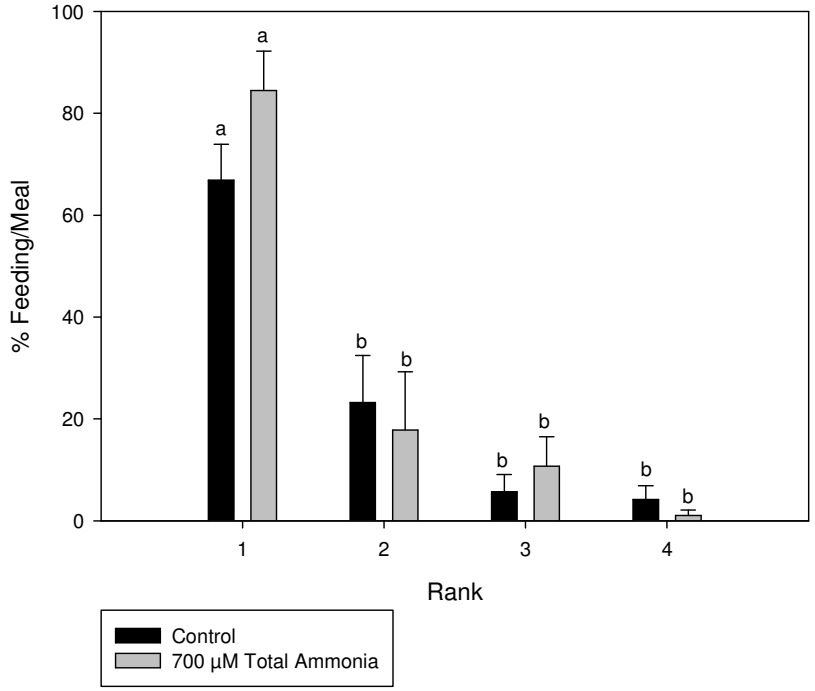
100
80
60
40
20
0
% Feeding/Meal
a
a
b
b
b
b
b
b
1
2
3
4
Rank
Control
700 µM Total Ammonia

Figure 6.

Figure 7. Oxygen consumption rates based on social status for control and 700 µM total ammonia hierarchies during period 1 and period 2. Values are means ± S.E.M. Different letters denote significant difference. Period 1: there is not a significant difference in oxygen consumption between control and 700 µM total ammonia hierarchies (p=0.056).There is no significant difference between ranks regardless of exposure (p=0.798). Period 2: there is no significant difference among control and 700 µM total ammonia hierarchies (p=0.209). There is also no significant difference among ranks regardless of exposure (p=0.268) (Control N=7; 700 µM total ammonia N=6; Two-way ANOVA; post-hoc test Fisher LSD).

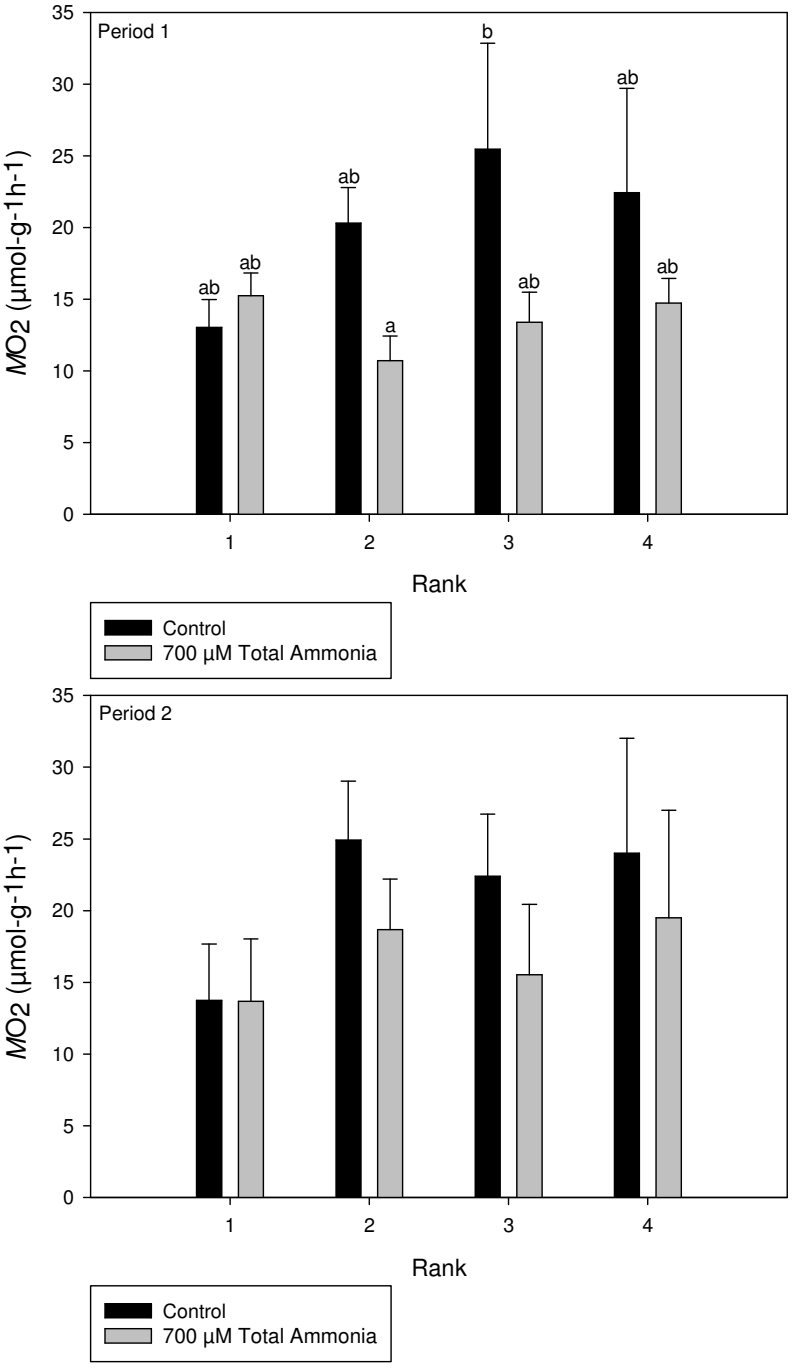
Period 1
MO2 (µmol-g-1h-1)
35
30
25
20
15
10
5
0
ab
ab
ab
a
b
ab
ab
ab
1
2
3
4
Rank
Control
700 µM Total Ammonia
Period 2
MO2 (µmol-g-1h-1)
Rank
Control
700 µM Total Ammonia

Figure 7.

Figure 8. Ammonia excretion rates based on social status for control and 700 µM total ammonia hierarchies during period 1 and period 2. Values are means ± S.E.M. Different letters denote significant difference. Period 1: there is no significant difference in ammonia excretion between control and 700 µM total ammonia hierarchies (p=0.160). There is also no significant difference among ranks regardless of exposure (p=0.671). Period 2: there is no significant difference between control and 700 µM total ammonia hierarchies (p=0.398). There is also no difference among ranks regardless of exposure (p=0.647) (Control N=7; 700 µM total ammonia N=6; Two-way ANOVA; post-hoc test Fisher LSD).

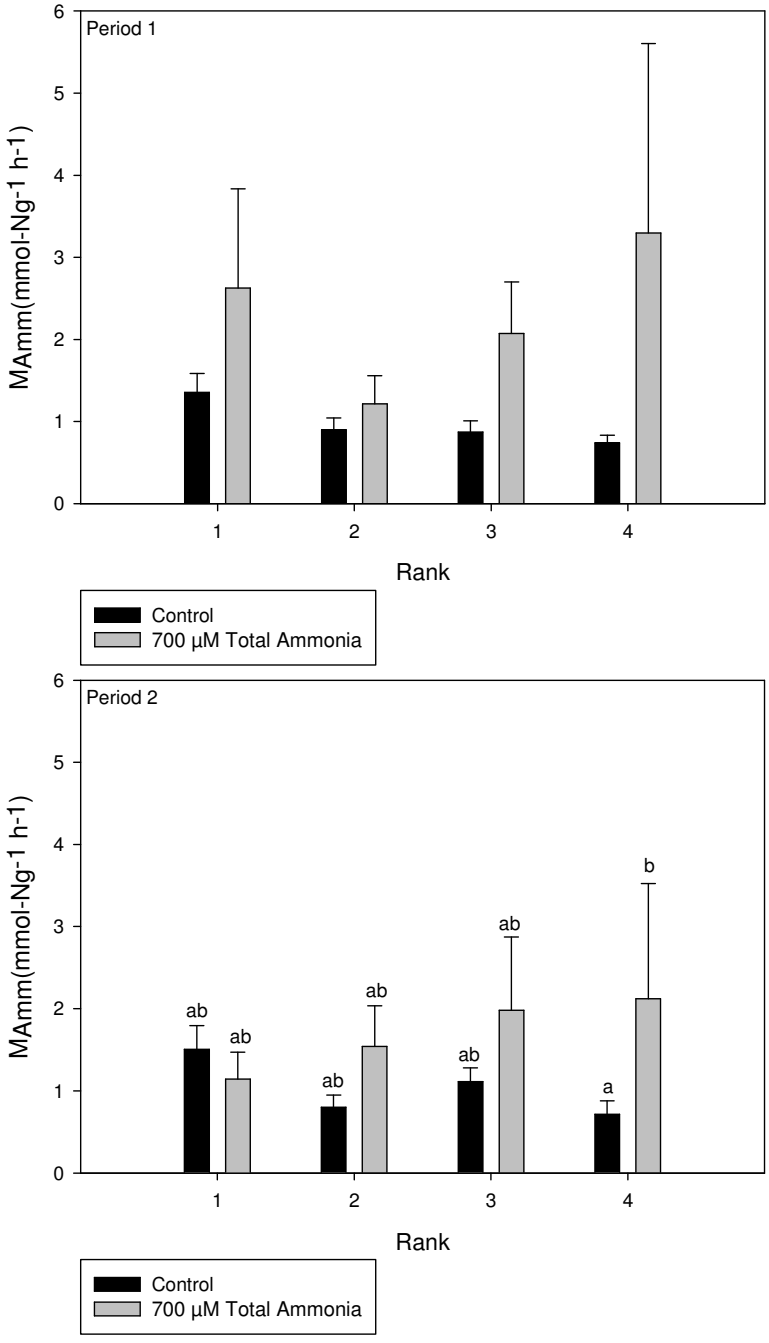
Period 1
MAmm(mmol-Ng-1 h-1)
Rank
Control
700 µM Total Ammonia
Period 2
MAmm(mmol-Ng-1 h-1)
ab
ab
ab
ab
ab
ab
a
b
Rank
Control
700 µM Total Ammonia

Figure 8.

Figure 9. Plasma cortisol based on social status for control and 700 µM total ammonia hierarchies. Values are means ± S.E.M. Different letters denote significant difference. There is a significant difference in plasma cortisol between control and ammonia hierarchies ($p=<0.001$). There is no difference among ranks, regardless of exposure ($p=0.287$). (Control N=6; 700 µM total ammonia N=6; Two-way ANOVA; post-hoc test Fisher LSD).

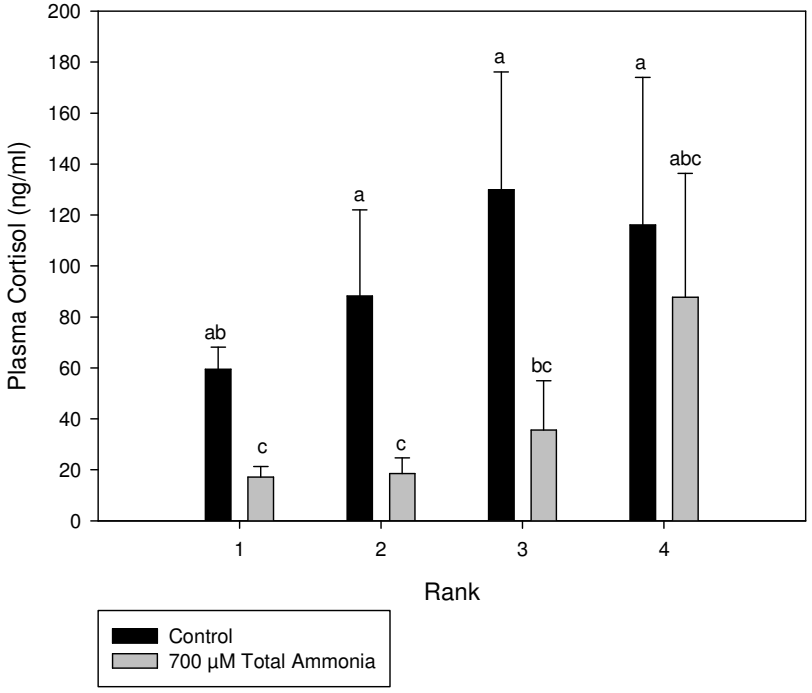
Plasma Cortisol (ng/ml)
200
180
160
140
120
100
80
60
40
20
0
ab
c
a
c
a
bc
a
abc
1
2
3
4
Rank
Control
700 µM Total Ammonia

Figure 9.

Figure 10. Plasma cortisol of fish held in 'dummy' feeding containers for various time periods. Values are means ± S.E.M.: N=4 per treatment. Different letters denote significant differences in plasma cortisol ($p = <0.001$; One-way ANOVA; post-hoc test Fisher LSD).

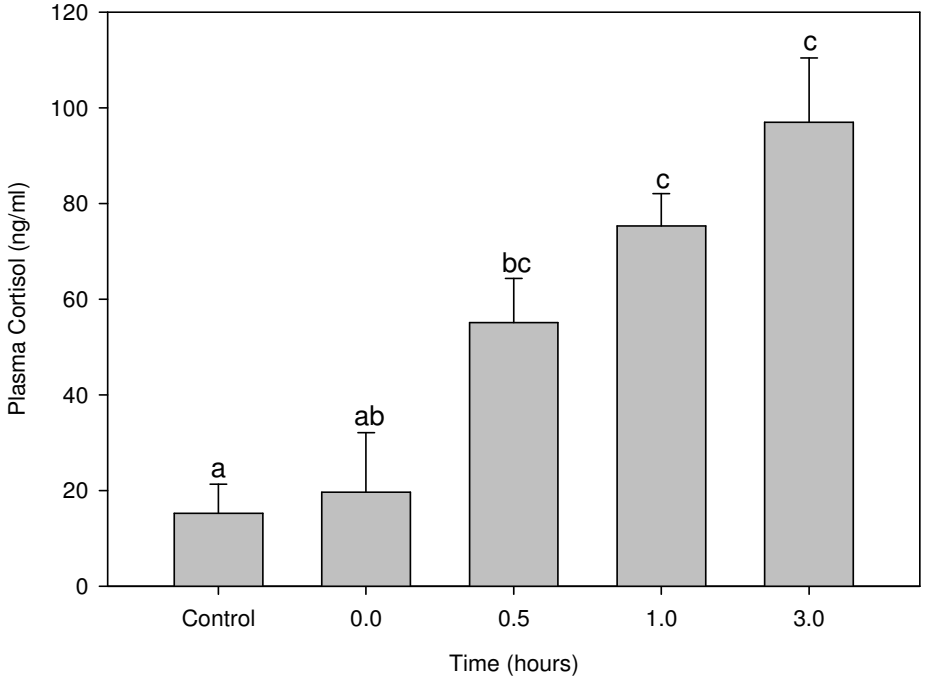
120
100
80
60
40
20
0
Plasma Cortisol (ng/ml)
a
ab
bc
c
c
Control
0.0
0.5
1.0
3.0
Time (hours)

Figure 10

Chapter 4: General Conclusion

Social status in a clean and highly competitive environment

Social hierarchies were formed in groups of four rainbow trout using a new method to deliver food. All of the groups created a stable hierarchy within 10 days, and all but one within the first five days. Each hierarchy consisted of a single dominant individual that displayed higher growth rate, higher condition factor, greater feeding consumption, higher ammonia excretion and higher protein utilization while having the lowest oxygen consumption compared to subordinates. All subordinates displayed similar physiology to each other. Dominant fish were seen as the 'winners' in these hierarchies while all the remaining fish became subordinates and were seen as the 'losers'.

From the results of Chapter 2, it appears that the social hierarchy created two distinct physiological groups based on social status: one dominant individual that displayed favorable physiological parameters, while the other three fish displayed similar, less favourable physiological parameters. This was different than what was originally hypothesized, as it was thought that each social status would have a distinct physiological profile, different from each other. It was believed that the fish within these hierarchies would be able to better compete amongst themselves, resulting in both distinctive physiology and distinctive aggression scores. Instead what was observed was one fish monopolizing food, having preferential physiology, while the other three were largely

excluded, manifesting similar less favourable physiologies despite distinctive aggression scores.

This, however, does fall in line with our current understanding of how rainbow trout hierarchies are established in the laboratory and the physiological profile of individuals that inhabit them (Sloman and Armstrong, 2002; Gilmour et al., 2005). These hierarchies consist of dominant and subordinate individuals, with dominant individuals being favoured. In a small setting, devoid of complexity such as in a laboratory arena, any limited resource can result in an intense display of aggressiveness due to the high competitiveness within the group. This intense competition leads to physiological divergent individuals, even in a short time span (24 hours in one laboratory study (Sloman et al., 2000)). Fish in these aggressive environments have two options: to become dominant or to become subordinate.

This, more than likely, does not occur in a natural environment due to the large, complex, constantly changing environment as well as the presence of other species co-habiting the same area. A common factor that laboratory studies cannot account for is the presence of predators. All of these factors will decrease aggression in natural fish populations meaning ‘dominant’ or ‘subordinate’ individuals cannot be easily identified. Increasing the number of fish within a hierarchy will also reduce the aggressiveness, as evident in the Appendix section. Social hierarchies consisting of seven individuals

displayed a different physiological profile than that observed in hierarchies of four rainbow trout.

What we can gain from laboratory studies conducted on salmonids (including the current one which looked at rainbow trout hierarchies) is that fish have the capability to form highly competitive social environments when given the opportunity. We can also assess the physiological consequences of this competition.

Individual aggression leads to differential physiology

When investigating individual behaviour, a continuum of physiological parameters based on aggressive acts was observed. Increased aggression was correlated with increased growth, increased condition factor, increased food consumption, increased ammonia excretion and increased protein utilization in aerobic metabolism. This overall pattern is probably due to aggressive acts having a direct effect on food consumption in this experimental design. This correlation between aggressive acts and physiological parameters became stronger when the specific level of aggression that occurred in each hierarchy was taken into account.

This finding, while not innately new, has important consequences. From observing a small fraction of the day (15 minutes each day representing 2% of the daylight fraction) over several days, it was possible to predict, somewhat confidently, the likely

physiological changes of individual fish. This shows it is possible to implement a non-invasive method to monitor the health of a group of fish.

Ammonia toxicity in establishing a social hierarchy

Chapter 3 investigated the effect of high environmental ammonia on the formation of social hierarchies and the physiology of individuals within these hierarchies. Total ammonia concentrations of 700, 1200, and 1500 µM total ammonia (2.97, 5.09 and 6.37 µM NH_3, respectively) were evaluated. These were higher than allowed by legislated water quality criteria for chronic ammonia exposures in Canada and the United States. I hypothesized that high, yet still sublethal, concentrations of ammonia would completely prevent a hierarchy from forming. Also, that a lower, but still elevated, external ammonia concentration would reduce appetite and swimming performance in dominant fish so that subordinate individuals would be healthier than in control hierarchies.

Exposure to 1200 and 1500 µM (5.09 and 6.37 µM NH_3) for 5 days resulted in such a dramatic reduction in aggression that a social hierarchy could not be determined. Indeed, no aggressive acts were recorded in the 1500 µM total ammonia groups. Thus the first hypothesis was confirmed. However, when trout were exposed to 700 µM total ammonia (2.97 µM NH_3), hierarchies still formed. The level of aggression recorded in this treatment was significantly lower than in control hierarchies, but higher than in 1200

and 1500 µM total ammonia treatments. The reduction in aggression is attributed to reduced swimming performance caused by high plasma ammonia.

The fact that a total ammonia concentration of 700 µM (2.97 µM NH_3) did allow a social hierarchy to form is a surprising result, since this value is nearly double the chronic Canadian guideline for protection (450 µM total ammonia or 1.03 µM NH_3). This attests to the level of competitiveness within a group of four rainbow trout in a laboratory setting. As stated earlier, in a non-complex environment, there are no factors that can limit the level of competiveness. At 1200 and 1500 µM total ammonia, which is three times higher than chronic protected guidelines, the fish were finally burdened enough by the high ammonia that a social hierarchy could not be established.

During period 1 (the first five days of the experiment), hierarchies exposed to 700 µM total ammonia (Ammonia hierarchies) displayed a significant reduction in growth and condition factor. This was probably due to reduced food consumption which has been previously documented to occur in rainbow trout exposed to high ammonia concentrations (Wicks and Randall, 2002; Oretega et al., 2005). However, food consumption was not measured during period 1 so this conclusion cannot be confirmed. As well, during period 1, Ammonia hierarchies tended to exhibit lower oxygen consumption than control hierarchies. This was attributed to reduced swimming performance limiting the metabolic demand of these fish.

However, these trends were reversed during period 2 as fish in Ammonia hierarchies displayed similar growth, condition factor, food consumption and oxygen consumption to fish in control hierarchies. It was proposed that biochemical and physiological changes occurred in Ammonia fish as they acclimated to the high ammonia environment. One possible mechanism is the increased activity of glutamate synthetase, which has been shown to effectively convert toxic ammonia to non-toxic glutamine and glutamate. Fish from the Ammonia hierarchies were then able to display similar physiology to those in the Control hierarchies. More long term studies are required in order to fully elucidate any possible changes in biochemical or physiological processes that might be occurring at this time.

It was envisioned that subordinates in the Ammonia hierarchies would be healthier than subordinates in control hierarchies because ammonia would have a larger effect on the dominant fish in Ammonia hierarchies. Dominant fish have the highest competitiveness and as such, it was believed they would be more susceptible to high external ammonia's effect on appetite and swimming performance.

This was not the case, as dominant individuals still displayed favourable physiological parameters (during period 1), much like dominant fish in control hierarchies. Subordinates displayed negative growth and condition factor during period 1 and these physiological parameters were not higher during period 2. Subordinates were seen as the 'losers' regardless of being in a clean or 'dirty' environment.

Non-invasive method for recording physiological parameters

The goal of using the non-invasive enclosure method was to collect as much data from the fish as possible without evoking a stress response. Nevertheless, the fish clearly mobilized cortisol during their confinement in the enclosure. This cortisol response was reflective of many previous studies in which salmonids have been shown to elevate plasma cortisol during capture, collection, or confinement (e.g. Ellis et al., 2004; Brydges et al., 2009). Cortisol has many effects on the physiology of fish (Gilmour et al., 2005), thus the response makes interpretation of physiological data more difficult. When conducting experiments, investigators normally wait until the fish has settled down (assuming that plasma cortisol has decreased to a stable baseline level) before taking a physiological measurement (several hours most of the time). However, such a strategy does not allow one to know the exact physiological parameters of the fish at a given time. It is also often assumed that the physiology displayed after the cortisol response is the same as before the cortisol response. This may not necessarily be the case.

While a non-invasive method has lofty goals, it requires considerable amount of time and effort both to establish it in the first place, and then to use it in collecting the data. As this thesis has shown, it is a very difficult approach to implement in rainbow trout. Nevertheless, despite its flaws, an important advantage of the non-invasive method

is that it allows for multiple samples from the same fish to be taken at different times, making it possible to track physiology over a time course (discussed below).

Ammonia in aquaculture and its influence on social hierarchies

In an aquaculture setting, social hierarchies and high ammonia levels are common problems that are detrimental to the overall fish health. However, this thesis work illustrates several factors that could be applied in aquaculture.

Groups of rainbow trout will form hierarchies consisting of dominant and subordinate individuals if given the opportunity, i.e. fighting for a limited resource. As mentioned in Chapter 1, there are several ways to reduce this, however, the very nature of fish husbandry (holding fish in a uniform environment where feeding occurs at predictable times and locations) leads to groups of fish forming social hierarchies. There are several ways to reduce this, such as, increased water flow and dispensing food at irregular times.

Also, monitoring fish for a period of time during the day by a non-invasive method could provide insights into individual fish health (mentioned previously). This becomes problematic when trying to identify hundreds or thousands of individual fish. Not only do you have to mark these fish in such a way that they will be identifiable months later (years in some cases), but the husbandry operations will need investment to make it

possible to view and record the fish. However, there are great benefits to overcoming these challenges, most notably, gaining an understanding of the physiological state of individual fish with minimal disturbance.

High density and static water conditions can cause ammonia build-up, which is a known toxicant to fish. What this thesis has illustrated is that if even in the face of high concentrations of ammonia, a social hierarchy can still form. But interestingly, fish appear to acclimate to these high ammonia levels, both physiologically and behaviorally, so that with time, normal levels of aggression and physiology are re-established As the high ammonia does reduce aggressive acts initially, but not chronically, this suggests that sublethal doses of ammonia might be incorporated into protocols for transporting fish or placing them into a new environment. Such strategies, together with the use of lower levels of ammonia to promote growth (Wood, 2004; Madison et al., 2009) , make ammonia a potentially useful tool for increasing aquacultural productivity.

Possible future work

As with any body of work, more questions were raised than were answered. Regarding the physiology of establishing a hierarchy, there are many questions left to be addressed. These include:

- Would analyzing physiological data based on social status and individual aggression in a hierarchy consisting of more than four individuals have the same conclusions as in a four individual hierarchy?
- What other physiological parameters would change based on social status (e.g. blood glucose, lactate, ions and the balance between aerobic versus anaerobic metabolism)?

Questions regarding the effect of high environmental ammonia on social hierarchies pose the most exciting areas for future exploration.

A suggestion for future work would be to investigate the effect of ammonia on social hierarchies that are already established and stable. It would be interesting to see if a small increase in ammonia concentration would result in a collapse of a hierarchy or would provide a benefit to fish of lesser social status, resulting in a 'rank switch'.

To further investigate the effect of poor water quality on the structure of social hierarchies, groups of fish could be exposed to an environment consisting of low dissolved oxygen and high ammonia, two stressors that may co-occur both in aquaculture and in natural environments. This would really test the strength of a social hierarchy. It would be interesting to monitor the response (both behaviourally and physiologically) of varying social status to this combined stressor.

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Appendix

Methods

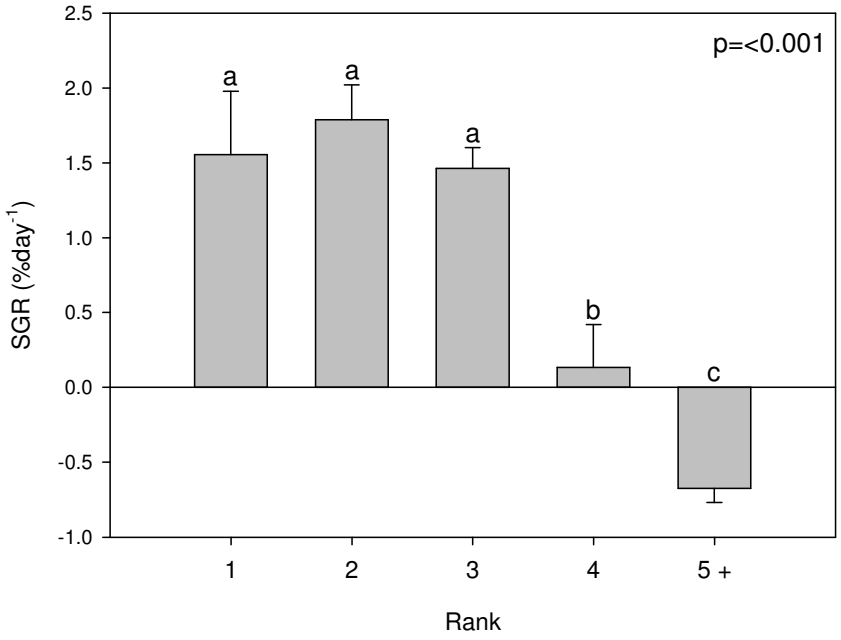
Formation of the hierarchy was accomplished by placing seven size-matched fish (12- 16 grams each, obtained from Humber Spring Trout Hatchery in Orangeville, ON) in a 60-L tank supplied with dechlorinated Hamilton tap water (12^{o}C), flow rate ~ 1 L min^{-1}, photoperiod of 12.5 h light: 11.5 h dark. Fish were anaesthetized individually in 0.08 g tricaine methanesulfonate (MS 222, neutralized) L $^{-1}$, weighed (to 0.01g) and measured for fork length (0.1 cm). Each fish was equipped with a PIT tag and uniquely branded to allow for individual identification. Branding was accomplished via a surgical probe dipped in liquid nitrogen. The cold tip was then pressed behind the head to form a distinctive mark. Fish were air-exposed for no more than 1 min and regained normal behaviour after two days, with feeding occurring five days after anaesthetization. No severe side-effects were observed from either procedure. Dominance was established by using the darkened feeding container (described previously) to deliver 2% repelleted fish food per day, twice a day (1% each feeding) over five days a week, for 3 weeks. Feeding container was left inside tank for 30 mins during which filming of feeding and aggressive behaviour occurred.

Aggression scores were recorded by videotaping the morning feeding. Scores were assessed based on aggression shown toward other fish and the resulting action of that fish. Aggressive behaviour (chases or approaches) were scored 1 point, while subordinate behaviours (chased or retreats) received 0. This created a dominance score

for each fish, and this dominance score was used to rank fish in descending order, meaning rank 1 was the most dominant individual and rank 7 was the least. After fish were ranked, those ranked 5 through 7 (i.e. the 3 most subordinate fish) were grouped together as rank 5 for data analysis.

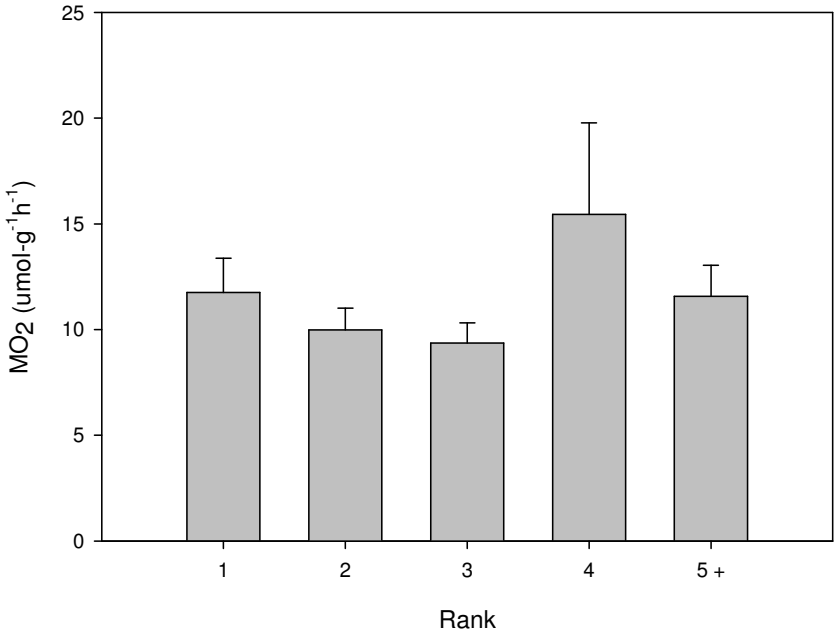
Fish were starved for 24 hours prior to recording physiological parameters (described previously). Each fish was measured twice, once every 7 days.

Appendix 1. Specific growth rate and social status of fish in a hierarchy in control water. Each hierarchy contained 7 individuals, rank 1 being the most dominant. Rank 5+ contains social status 5, 6 and 7. Different letters denote significant differences. Values are means ±S.E.M :N=4 ($p=<0.001$, One way ANOVA; post-hoc test Student-Newman-Keuls).



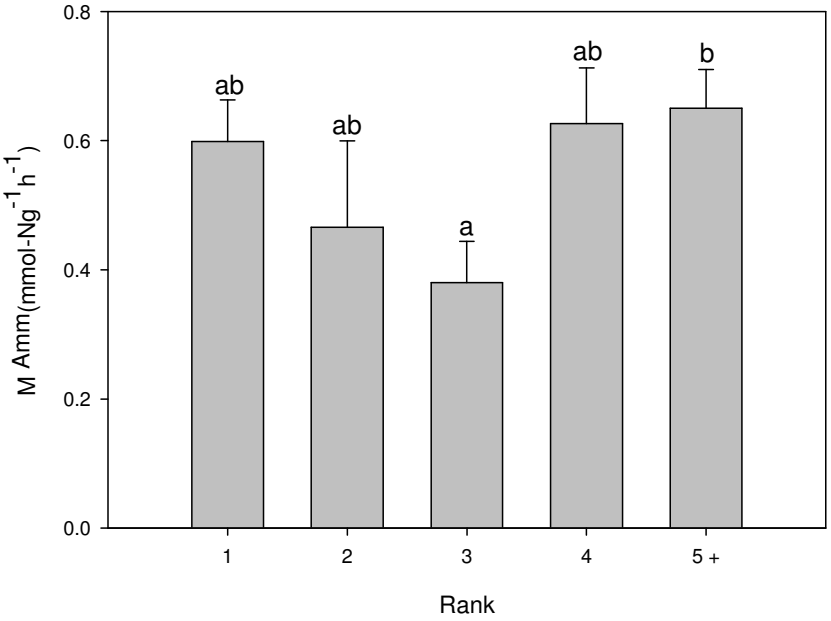
Appendix 1.

Appendix 2. Oxygen consumption rate and social status of fish in a hierarchy in control water. Each hierarchy contained 7 individuals, rank 1 being the most dominant. Rank 5+ contains social status 5, 6 and 7. Values are means ± S.E.M.: N=4 ($p=0.470$, One way ANOVA).



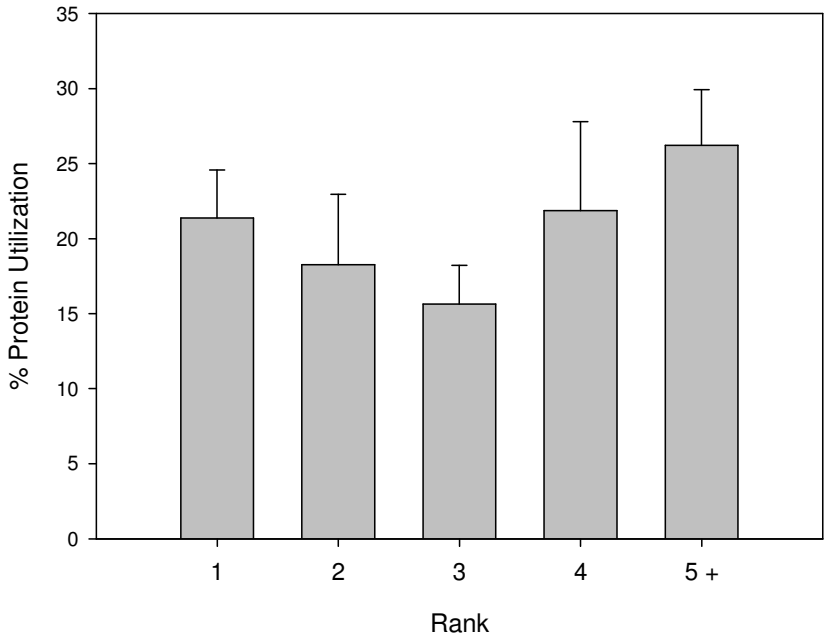
Appendix 2.

Appendix 3. Ammonia excretion rate and social status of fish in a hierarchy in control water. Each hierarchy contained 7 individuals, rank 1 being the most dominant. Rank 5+ contains social status 5, 6 and 7. Different letters denote significant differences. Values are means S.E.M.: N=4 (p=0.180, One way ANOVA; post-hoc test Student-Newman-Keuls).



Appendix 3.

Appendix 4. Protein utilization and social status of fish in a hierarchy in control water. Each hierarchy contained 7 individuals, rank 1 being the most dominant. Rank 5+ contains social status 5, 6 and 7. There are no significant differences between values. Values are means S.E.M.: N=4 ($p=0.595$, One way ANOVA).



Appendix 4.