Causes of intra-specific variation in metabolic rate in zebrafish, *Danio rerio*

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ABSTRACT

Many studies have reported individual differences in resting metabolic rate (RMR), the energetic cost of self-maintenance. Differences among individuals in the energetic cost of self-maintenance may influence life-history decisions and hence, fitness. In this study, we examined potential causes of intra-specific variation in RMR in zebrafish, *Danio rerio*. First, the repeatability of RMR was determined to check whether a single measure was reflective of future physiological performance. As predicted, RMR was repeatable over a period of three weeks. However, none of stress-coping style, baseline cortisol levels, metabolically-active organ (gill, heart, intestine and liver) mass, aggression or activity levels were correlated with RMR, i.e. none of these factors were significant contributors to individual variation in RMR. These results imply that other factors must be sought to explain the inter-individual variation in RMR observed in zebrafish.

RESUMÉ

Plusieurs études ont signalé des différences entre individus dans le taux métabolique au repos (TMR), soit le coût énergétique d'auto-entretien. Les différences individuelles du coût énergétique d'auto-entretien peuvent influencer les décisions en lien aux traits d'histoire de vie et ainsi, l'aptitude phénotypique de l'individu. Dans cette étude, nous avons examiné des facteurs potentiels pouvant causer la variation intra-spécifique du TMR des poissons zèbres, *Danio rerio.* Tout d'abord, la répétabilité du TMR fut déterminée dans le but de vérifier si une mesure unique du TMR pouvait refléter la performance physiologique future. Comme prédit, le TMR fut répétable sur une période de trois semaines. Cependant, aucune corrélation entre soit le syndrome de gestion de stress, le niveau basal de cortisol, la masse des organes métaboliquement actifs (branchies, cœur, intestin et foie), les niveaux d'agression et d'activité et le TMR fut établie, c'est-à-dire que ces facteurs ne contribuait de façon significative à la variation individuelle du TMR. Ces résultats suggèrent que d'autres éléments doivent être examinés afin d'expliquer les différences intra-spécifiques du TMR observées chez les poissons zèbres.

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LIST OF ABBREVIATIONS

AAS – Absolute aerobic scope

ACTH – Adrenocorticotropic hormone

AMR – Active metabolic rate

αwO_2 – Solubility coefficient of oxygen in fresh water

BMR – Basal Metabolic Rate

CO_2 – Carbon dioxide

Dom – Dominance

EIA – Enzyme immunoassay

HPA – Hypothalamic-Pituitary-Adrenal axis

HPI – Hypothalamic-Pituitary-Interrenal axis

MIS – Mirror Image Stimulation

$\dot{M}O_2$ – Rate of oxygen consumption

MR – Metabolic rate

N – Sample size/number of animals

O_2 – Oxygen

ΔPO_2 – Difference between initial and final PO_2

PO_2 – Partial pressure of oxygen

PwO_2 – Partial pressure of oxygen in water

PwO_{2i} – Initial partial pressure of oxygen in water

PwO_{2f} – Final partial pressure of oxygen in water

r – Pearson's product-moment correlation

rRMR – Residual resting metabolic rate

rSMR – Residual standard metabolic rate

RMR – Resting Metabolic Rate

SMR – Standard Metabolic Rate

vol_c – Volume of water in the chamber

CHAPTER ONE:

GENERAL INTRODUCTION

1.1 Importance of metabolic rate in fitness

Metabolic rate refers to the rate at which an animal oxidizes substrates to produce energy. Most animals have a finite amount of energy available and must allocate it effectively among the different components of survival, which include growth and self-maintenance, and reproduction (Stearns, 1992). When faced with challenges in the environment, organisms must prioritize the distribution of energy. These animals are confronted with life-history trade-offs that force them to choose between growth and survival (Lima, 1998; Biro et al., 2004; Biro et al., 2006; Stamps, 2007) or fecundity (Zera and Harshman, 2001; Reid et al., 2003; Pettay et al., 2005; Nussey et al., 2006). This choice may be influenced by genetic factors, maternal effects and early life experiences (Stamps, 2007). The choice often boils down to the adoption of a life-history pattern that favours fast growth and early maturation versus a life-history strategy of slower growth with later maturation (Thorpe et al., 1998). Morphological and/or physiological specializations that favour one strategy over the other may follow. For example, an individual may possess a large heart or gill/lung surface to support a high metabolic rate that in turn may be needed for fast growth, or may invest in early gonadal development for early maturation (Huntingford et al., 2012). Because metabolic rate can influence growth, it is expected to be linked to life history trade-offs and hence, the fitness of an animal (Stearns, 1992; Johnston et al., 2007; Careau et al., 2008; Burton et al., 2011). However, behavioural and physiological traits that support high growth rates also may increase the risk of mortality (Arendt, 1997; Mangel and Stamps, 2001; Biro et al., 2006). For example, larval amphibians that foraged and grew at higher rates also faced a higher risk of predation than less active conspecifics (Werner and Anholt, 1993; Sih et al., 2004b). This example is indicative of the fitness costs and benefits that are associated with

early versus later maturation (or fast versus slow growth), and may explain why these life-history strategies have coexisted over evolutionary time (Huntingford et al., 2012).

Resting energy metabolism may be the most relevant means of judging an organism's fitness because it reflects the minimum, necessary energy cost required for self-maintenance. With a finite amount of energy available, individuals that spend the least for self-maintenance should have the capacity to allocate more towards growth and reproduction, a concept termed the 'compensation' hypothesis (Burton et al., 2011). Other studies have proposed the 'increased intake' hypothesis, whereby individuals with high resting metabolism are predicted to possess greater fitness owing to larger internal organs (Chappell et al., 2007) and higher maximum metabolic rates (Chappell et al., 2007; Biro and Stamps, 2010). Such enhanced 'metabolic machinery' (Biro and Stamps, 2010) allows for higher sustained energy throughput, thus enabling greater assimilation of energy for growth and reproduction (McNab, 1980). In ectotherms, standard metabolic rate (SMR) is the lowest rate of metabolism measured at a particular temperature in inactive, post-absorptive individuals. Basal metabolic rate (BMR) is the equivalent measurement of metabolism in endotherms, where the cost of endothermy also applies (McNab, 2002). Measurement of BMR in endotherms is conducted at temperatures within the thermoneutral zone. Both BMR and SMR refer to an organism's minimum cost of maintenance (Brody 1945), and include the cost of processes such as maintenance of the mitochondrial H^+ gradient, protein turnover, ion transport, hormone production, blood circulation and ventilation (Hulbert and Else, 1981; Hochachka and Guppy, 1987; Bennett, 1988; Rolfe and Brown, 1997; Hulbert, 2000). Because measurement of SMR or BMR requires that the animal be absolutely still, something that is difficult to achieve in practice, many studies use instead resting metabolic rate (RMR). Resting metabolic rate is a measure of the rate of energy metabolism that

is applied to either endotherms or ectotherms, in a post absorptive state, and accommodates low levels of spontaneous activity.

Many studies have suggested that RMR influences fitness-related traits, although empirical evidence in support of this hypothesis remains mixed (reviewed by Burton et al., 2011). Assessments of fitness often encompass measurements of growth, reproductive output (number of offspring produced), reproductive fitness (number of surviving offspring), senescence and/or survival/lifespan. The relationship between RMR and growth may depend on food availability (Alvarez and Nicieza, 2005; Steyermark et al., 2005). Under *ad libitum* food conditions, individuals of high RMR demonstrated higher growth rates, a result that supports the 'increased intake' hypothesis. However, under 'natural' conditions, where the food supply was restricted, individuals of high RMR did not grow faster than conspecifics of low RMR and in some cases even lost mass (Killen et al., 2011). A similar trend was found for relationships between RMR and survival (reviewed by Burton et al., 2011). Even in the face of such variable data, it is clear that relationships can exist between RMR and life history traits, and in turn these relationships imply that the fitness of an organism can be influenced by its RMR.

1.2 Hypothesis and Predictions

If metabolic rate is capable of influencing fitness-related traits, then there is likely to be variation in metabolic rate among individuals, and this variation will be associated with differences among individuals in aspects of their physiology and/or behaviour. The objective of the present study was to investigate potential causes of intra-specific variation in metabolic rate in zebrafish, *Danio rerio*. An underlying assumption of such studies is that metabolic rate is characteristic of each individual and hence can be reliably measured over time, i.e. the relative

RMR rankings of a group of fish should remain consistent over time. If RMR is not repeatable over a period of time, then a single measure of metabolic rate may not be representative of future physiological performance. Thus, an initial step in the present study was to test the repeatability of RMR over time in zebrafish. As in other similar studies (McCarthy, 2000; Maciak and Konarzewski, 2010; Norin and Malte, 2011), we predicted that metabolic rate would be an individual characteristic that exhibits repeatability for a period of time. After evaluating the repeatability of metabolic rate, the capacity of several physiological and behavioural factors to account for inter-individual variation in RMR was assessed. The factors investigated were stress coping style, baseline cortisol concentrations, organ mass, and the personality traits of aggression and activity, and were chosen based on previous studies that suggested the possibility of a link with RMR; this background information is reviewed below. In brief, based on previous studies (Mueller and Diamond, 2001; Huntingford et al., 2010; Martins et al., 2011), we predicted that individuals that demonstrated a proactive stress coping strategy would also exhibit a higher RMR than their reactive conspecifics. Previous studies documented a positive effect of cortisol administration on metabolic rate (Barton et al., 1987; Morgan and Iwama, 1996; De Boeck et al., 2001) and we therefore predicted that individuals with higher basal cortisol levels also would exhibit higher RMR. The mass of active organs such as the small intestine, kidney, heart and liver was found to account for over half of the variation in RMR in mice (Konarzewski and Diamond, 1995). Hence, we predicted that individual zebrafish with larger energetically-demanding organs would have higher RMR. A recent area of intensive research effort has linked personality traits to metabolic rate (Careau et al., 2008; Biro and Stamps, 2010). Based on this work, we predicted that fish demonstrating higher levels of aggression and activity would have higher RMR.

1.3 Assessment of metabolic rate

Among the techniques available for the measurement of RMR (e.g. calorimetry, bomb calorimetry, doubly-labelled water and respirometry), respirometry involving measurement of the rate of oxygen consumption was deemed to be most appropriate for the purposes of the present study (reviewed by Cech, 1990). In this approach (Steffensen, 1989; see Cech, 1990), the fish is housed in an appropriate chamber and the rate at which the fish removes oxygen from the water in the chamber is measured. The respirometer may be closed, open, or intermittently closed. In closed-system respirometry, there is no replacement of water in the respirometry chamber during the period of measurement, and the rate at which the fish depletes water O_2 is measured by monitoring dissolved O_2 levels or the partial pressure of O_2 in the water (taking into account the solubility of O_2 in water; Boutilier et al., 1984). Circulation of water within the respirometer, typically with a pump, ensures mixing. Open or flow-through respirometry involves the measurement of O_2 in the water flowing into and out of the respirometer. Water flow rate through the respirometer must be adjusted to create a sufficient difference (i.e. a difference that can be reliably measured) in O_2 concentration between the inflowing and outflowing water. Particularly for low rates of O_2 consumption, achieving an appropriate difference in O_2 concentration may result in overly slow flow of water through the respirometer [see Steffensen, (1989) for a detailed description of the sources of error in open respirometry]. With intermittent-flow respirometry, relatively brief periods of closed-system respirometry are interspersed with flushing of the respirometer to avoid excessive depletion of water O_2 and/or accumulation of CO_2 and other waste products (Steffensen, 1989). Although intermittent-closed system respirometry is generally considered to be the preferred approach (Steffensen, 1989),

Cech (1990) pointed out the need to avoid disturbance of the fish during the period of flushing the respirometer, something that is particularly important in the measurement of RMR.

1.4 The repeatability of measurements of metabolic rate

Determining whether repeated measures of RMR over time would yield comparable values was a key first step in the present study. If measurements of RMR are repeatable, then a single measure of RMR should reflect future physiological performance. Although RMR is widely assumed to be characteristic of the individual (McCarthy, 2000; Cutts et al., 2001; Maciak and Konarzewski, 2010), relatively few studies have tested this assumption explicitly. Where this assumption has been tested, the data suggest that RMR exhibits temporal repeatability but only for relatively short periods of time. For example, McCarthy (2000) reported a high degree of repeatability of RMR in rainbow trout, *Oncorhynchus mykiss*, over a period of 113 days. Similarly Arctic char, *Salvelinus alpinus* (Cutts et al., 2001) and spined loach, *Cobitis taenia* (Maciak and Konarzewski, 2010) exhibited repeatability of SMR over 6 and 5 month periods, respectively. In a study performed on young brown trout, *Salmo trutta* L., the repeatability of measurements of SMR, active metabolic rate (AMR) and absolute aerobic scope (AAS; the difference between AMR and SMR) was found to gradually disappear over a 15 week period (Norin and Malte, 2011). These authors suggested that the gradual decline in repeatability could be attributed to use of a restricted feeding regime. Supporting this possibility, O'Connor et al. (2000) reported that overwintering juvenile Atlantic salmon (*Salmo salar*) parr maintained the same rank order for three measures of rSMR (relative standard metabolic rate) through a three month period where food supply was unlimited. Because zebrafish used in the present study were provided with an abundant supply of food, we predicted that metabolic rate

would remain consistent over a three week period. This period of time was chosen to correspond with the time required for experiments testing potential causes of variation in RMR.

1.5 Potential causes of intra-specific variation in RMR

Historically, in an approach dubbed "the tyranny of the golden mean" (Bennett, 1987), noise or inconsistency in metabolic rate among individuals was attributed to instrument error rather than actual biological variation. Recent advancements and refinements in the equipment used for respirometry suggest that equipment- and calculation-based errors should account for only 5-10% of the variation observed in metabolic rate among individuals (Speakman et al., 2004). This implies that observed differences in metabolic rate and daily energy expenditure may be caused by biological variation. Several 'extrinsic' and 'intrinsic' factors, i.e. environmental factors versus individual traits, that could potentially contribute to variation in metabolic rate among individuals have been identified (Careau et al., 2008; Burton et al., 2011; Norin and Malte, 2012; Konarzewski and Ksiazek, 2013).

1.5.1 Extrinsic factors

Intra-specific variation in metabolic rate can be caused by a number of physical and biological factors that may be present in the environment that the organism inhabits (Careau et al., 2008; Burton et al., 2011). Prior studies have shown that external factors such as temperature, challenges to the immune system and conspecific density during early developmental stages can influence RMR in adulthood (Steyermark and Spotila, 2000; Ots et al., 2001; Freitak et al., 2003; Careau et al., 2010; Le Lann et al. 2010). In birds, RMR was influenced by brood density during early development. A study on zebra finches (*Taeniopygia guttata*) revealed that large brood size

increased the metabolic rate of 1-year old birds (Speakman et al., 2004), while in passerine birds, a higher metabolic rate was observed in individuals that were raised in smaller broods (Burness et al., 2000). In insects (Freitak et al., 2003), birds (Ots et al., 2001) and mammals (Careau et al., 2010), upregulation of immune function induced by challenges to the immune system also increased RMR. For example, in a study on juvenile eastern chipmunks (*Tamias striatus*) infected with botfly larvae, RMR was found to increase with parasite load and these differences persisted into adulthood (Careau et al., 2010). This increase in RMR was attributed to the increased energy demand brought on by parasites. Exposure to parasites during development was thought to permanently up-regulate certain components of the immune system, thereby increasing the basic cost of maintenance, or metabolic rate (Careau et al., 2010). In insects, SMR increased after wounding, a response attributed to the production of immune peptides to ward off infection from microorganisms (Freitak et al., 2003). In birds such as the great tit (*Parus major*), a 9% increase in metabolic rate was observed for individuals mounting an antibody response to foreign antigens (Ots et al., 2001).

A study performed on rats reported that the quality of food provided during early development affected metabolic rate by influencing traits such as organ size, nutrient metabolism and enzyme physiology (Desai and Hales, 1997). Variation in food quality was found to potentially alter the programming of liver metabolism by permanently changing the activity of key hepatic enzymes involved in glycolysis and gluconeogenesis (such as glucokinase and phosphoenolpyruvate carboxykinase) (Desai and Hales, 1997). In other words, low-quality nutrition was responsible for setting the liver into a starved state. Food consumption also can affect RMR in mature organisms. For example, food deprivation caused a reduction in SMR of Atlantic salmon (O'Connor et al., 2000) perhaps as a strategy to down-regulate metabolic rate

during periods of low food availability (Hickman 1959; Brett, 1965; Muir et al., 1965; Hepher et al., 1983). Re-feeding increased SMR, supporting the idea that this transient reduction in SMR may be a mechanism for energy conservation during periods of food deprivation.

In the present study, zebrafish were generally maintained under conditions that promoted healthy growth. Moreover, it was assumed that all fish experienced similar conditions during early development because the adult fish used in the present study were raised by a breeder (not captured from the wild). Therefore, we expected the contributions of extrinsic factors to variation in RMR to be minor and focused instead on intrinsic factors that could contribute to individual variation in RMR.

1.5.2 Intrinsic factors

Burton et al. (2011) identified three types of intrinsic factors that may contribute to individual variation in RMR: local adaptation, heritability and genetic determinants; maternal contributions; and biochemical, physiological and behavioural factors. Genetic differences among individuals, including those resulting from local adaptation, could provide one source of variation in RMR. For example, studies performed on spatially-distributed populations of the isopod (*Porcellio laevis*) demonstrated that inter-population differences in RMR existed even in F_1 generation offspring that were bred and reared in a common environment (Lardies and Bozinovic, 2008). Although the heritability of RMR has been found to be low in most studies to date (Nespolo et al., 2003; Nespolo et al., 2005; Ronning et al., 2007; Ketola and Kotiaho, 2009), selective breeding experiments performed on wild rodents (Sadowska et al., 2005) and blue tits, *Cyanistes caeruleus* L. (Nilsson et al., 2009), indicated that RMR can respond to selection, which provides evidence that RMR could be a heritable trait (Burton et al., 2011).

Several recent studies provided evidence that offspring RMR can be influenced by maternal contributions of hormones to eggs. For example, experimental elevation of testosterone in zebra finch eggs resulted in elevated RMR in offspring that were observed to persist into adulthood (Tobler et al., 2007; Nilsson et al., 2011). Female three-spined stickleback (*Gasterosteus aculeatus*) exposed to the threat of predation produced eggs with elevated cortisol levels; in turn, these eggs yielded offspring of elevated RMR (Giesing et al., 2011). A similar effect was reported in brown trout, *Salmo trutta*, eggs exposed to experimentally-elevated cortisol levels (Sloman, 2010). Hence, maternal hormone contributions can impact offspring RMR. In mature animals, individual variation in hormone levels also is thought to contribute to individual variation in RMR (Buchanan et al., 2001; Chastel et al., 2003; Ros et al., 2004). For example, inter-individual variation in BMR in a population of free-living house sparrows (*Passer domesticus*) was attributed to differences in plasma tri-iodothyronine (T3) levels (Chastel et al., 2003). In a separate study on male house sparrows, increases in testosterone were shown to be associated with increases in BMR (Buchanan et al., 2001). In Mozambique tilapia (*Oreochromis mossambicus*), body mass-corrected routine metabolism was positively correlated to 11-ketotestosterone levels (Ros et al., 2004).

The glucocorticoid stress hormone cortisol must be considered as a candidate hormone for explaining individual variation in RMR because this hormone has significant effects on a variety of metabolic and physiological functions (reviewed by Wendelaar Bonga, 1997; Mommsen et al., 1999). For example, cortisol plays a major role in influencing intermediary metabolism (Vijayan et al., 1994; Vijayan et al., 1996; Vijayan et al., 1997), ionic and osmotic regulation (McCormick, 1995), and immune function (Wendelaar Bonga, 1997). Responses to stressors are energy demanding processes and have been found to be associated with increases in

metabolic rate in fish (Barton et al., 1987). To cope with the increase in energy demand, substrates have to be mobilized to fuel cellular processes. Cortisol plays a role in increasing circulating glucose concentrations in the blood by stimulating glycogenolysis (Mommsen et al., 1999). For example, intraperitoneal implants of cortisol were found to elevate oxygen consumption and plasma glucose levels in cutthroat trout (*Oncorhynchus clarki clarki*) (Morgan and Iwama, 1996). The links between cortisol levels and fitness also have been investigated, with most studies reporting a negative correlation between fitness and baseline cortisol levels (reviewed by Bonier et al., 2009). This relationship, also known as the 'Cort-Fitness' hypothesis, is based on two central ideas. The first states that environmental challenges usually elicit an increase in baseline cortisol levels. High cortisol levels favour the reallocation of resources from reproduction towards self-maintenance. Therefore, this effect implies a decrease in fitness with increasing environmental challenges, which is the second central tenet of the 'Cort-Fitness' hypothesis (Bonier et al., 2009). Despite the links that have been reported between stress responses and metabolic rate, the possibility of a link between baseline glucocorticoid levels and RMR in fish has not yet been investigated. In the present study, the hypothesis that RMR is determined at least in part by baseline circulating cortisol levels was tested. Based on the literature for the effects of elevated cortisol or stress responses on metabolic rate, we predicted that higher baseline glucocorticoid levels in mature zebrafish would be associated with higher RMR.

The RMR of individuals may be affected by factors such as protein turnover, gluconeogenesis, enzyme activity, nitrogenous waste synthesis, and proton transport across the membranes of mitochondria during energy metabolism (Rolfe and Brown, 1997). Differences in the cost of operation of these fundamental processes may contribute to the variation in RMR

among individuals. Studies performed on birds and rodents have reported that differences in the sizes of kidney, liver, intestines and heart accounted for more than half of the variation in RMR (Biro and Stamps, 2010). Similarly, leopard frogs (*Rana pipiens*) with high RMR also possessed relatively larger kidneys (Steyermark et al., 2005), while Andean toads (*Bufo spinulosus*) with high RMR showed signs of having larger kidneys, intestines, liver and hearts (Naya et al., 2009). Larger or high capacity metabolic engines can process food faster and/or more efficiently. For example, Atlantic salmon of high RMR were able to digest food more quickly than conspecifics of lower RMR (Millidine et al., 2009). In mice (*Mus musculus*), a decrease in temperature (from 23^{o}C to 5^{o}C) forced an increase in food consumption among individuals. However, only those individuals with a relatively high RMR maintained a high digestive efficiency during cold treatment because they possessed larger metabolic machines that were able to cope with the increased food uptake and energy demand (Ksiazek et al., 2009). These studies suggest that individuals with larger organs are more likely to possess higher RMR, owing to the high energy costs associated with basal maintenance of large organs (Biro and Stamps, 2010).

The relationships among RMR, metabolic engine 'size' and energy output largely have been examined in mammals (Konarzewski and Diamond, 1995; Speakman and McQueenie, 1996; Selman et al., 2001; Chappell et al., 2007) and birds (Daan et al., 1990; Chappell et al., 1999; Weber and Piersma, 1996). In several cases, organ size differences were found to influence differences in metabolic rate among individuals. For example, variation in the size of the intestine, liver, kidney and heart contributed 52% of the variation in metabolic rate in mice (Konarzewski and Diamond, 1995). Fewer studies have examined the relationship between organ mass and RMR in ectothermic vertebrate species such as amphibians (Garland, 1984; Garland and Else, 1987), reptiles (Steyermark et al., 2005) and fish (Norin and Malte, 2012). Biro and

Stamps (2010) suggested investigating the influence of the gill in supporting energy metabolism in aquatic animals. This highly metabolically-active organ takes part in key functions that include gas exchange, acid-base balance and ionic/osmotic regulation (Evans et al., 2005). Mitochondrion-rich (MR) cells located in the gill epithelium are key sites for active ion transport and are responsible for maintaining ionic/osmotic gradients through processes that are energy consuming (Hirose et al., 2003; Evans et al., 2005). To keep up with the energy demand of these processes, specific cells in the gill have been identified as sites of glycogenesis and glycogenolysis (Tseng et al., 2007). In tilapia (*Oreochromis mossambicus*) for example, transfer to saltwater caused glycogen-rich cells in the gills to accumulate glycogen and liberate energy, through glycogenolysis, for adjacent MR cells to use for ion secretion (Chang et al., 2007). Based on these studies, we predicted that the mass of metabolically-active organs such as the gill, heart, intestine and liver would be correlated with RMR.

Resting metabolic rate also may be related to particular types of behaviour recently identified as "personality traits". Animal personalities or behavioural syndromes are defined as suites of behavioural traits that co-vary across contexts or situations (Sih et al., 2004a). Animal personalities have provoked interest because plasticity in behavioural traits may be limited, preventing the animal from behaving in an optimal fashion in all situations. For example, an individual that demonstrates high levels of aggression towards prey is likely to be aggressive towards conspecifics or predators, situations in which aggression may be less appropriate (Sih et al., 2004a). Fives axes of animal personality have been identified (Reale et al., 2007); shyness-boldness, exploration-avoidance, activity, aggressiveness and sociability. Consistent differences in these personality traits have been observed among individuals and are considered to be inherent to the individual (Reale et al., 2007).

TABLE 1. Relationships between RMR and various behaviours studied in fish. Positive (+), negative (-), and non-significant relationships (ns) between RMR and the studied behavioural traits have been indicated [data extracted from Table 1 in Biro and Stamps (2010)].

Fish (Species name)	Behaviour	Relationship with RMR	Ref.
Salmon (*Salmo salar*)	Aggression	+	Cutts et al. (1998)
Trout (*Oncorhynchus mykiss*)	Dom: Access to food and feeding site	+	McCarthy (2001)
Salmon (*Salmo salar*)	Dom: Access to food and feeding site	+	Metcalfe et al. (1995)
Arctic charr (*Salvelinus articus*)	Dom: Access to food	+	Cutts et al. (2001)
Salmon (*Salmo salar*)	Dom: Access to food and feeding site	+	McCarthy (2001)
Salmon (*Salmo salar*)	Boldness	+	Finstad et al. (2007)
Salmon (*Salmo salar*)	Aggression	+	Cutts et al. (1999)
Salmon (*Oncorhynchus masou*)	Dom: Access to feeding site	+/+	Yamamoto et al. (1998)
Carp (*Cyprinus carpio*)	Boldness	+	Huntingford et al. (2010)
Brook charr (*Salvelinus fontinalis*)	Activity	NS	Farwell and McLaughlin (2009)

*Dom – Assessment of dominance

Differences in personality traits may impact an organism's fitness by affecting its risk of predation (Reale and Festa-Bianchet, 2003; Bell and Sih, 2007), its ability to compete for mates and food (Dingemanse et al., 2004) or its response to social challenges (Dingemanse and Reale, 2005; Sinn et al., 2006). Traits such as aggression, boldness and activity also are capable of affecting an animal's ability to acquire and defend food resources (Careau et al., 2008). This, in turn, will affect an individual's daily energy expenditure and hence, fitness. Correlation among different personality traits leads to formation of a behavioural syndrome. For example, individuals that demonstrate greater activity are also likely to show higher levels of boldness, aggression and exploratory behaviour (Biro and Stamps, 2008). Hence, behavioural syndromes provide a method of studying animal personality traits as a package rather than as individual, isolated units. Stress-coping style is an example of a behavioural syndrome in which aggression and activity both figure.

1.5.2a Stress coping styles

Individuals of a particular species may respond to a stressor in different manners. Identification of consistent response to a stressor led to the definition of stress-coping styles as "*a coherent set of behavioural and physiological stress responses, which is consistent over time and which is characteristic to a certain group of individuals*" (Koolhaas et al., 1999). Two opposing styles are generally recognized, proactive and reactive (Koolhaas et al., 1999). Proactive individuals demonstrate high levels of active avoidance, aggression, boldness, behavioural inflexibility (i.e. tendency to form behavioural routines) and attempts to counteract the stressful stimulus. In contrast, reactive individuals showed immobility, lowered aggression and flexibility in behaviour when exposed to a stressor. Proactive individuals also demonstrate

lower activity of the hypothalamic-pituitary-adrenal (HPA; or hypothalamic-pituitary-interrenal, HPI, in fish) stress axis than reactive individuals resulting in lower circulating glucocorticoid stress hormone concentrations in response to a stressor. Proactive individuals also demonstrated a higher sympathetic response to a stressor than reactive conspecifics (Koolhaas et al., 1999; Overli et al., 2007). Evidence of stress coping styles has been reported for a number of teleost fish including cichlids, sticklebacks, salmonids and a variety of other tropical species (Overli et al., 2007; reviewed by Huntingford et al., 2012). For example, 'high' and 'low' responding lines of rainbow trout were generated by individually selecting fish for consistently high or low post-stress cortisol levels, respectively (Pottinger and Carrick, 2001). The behavioural traits demonstrated by low-responders were typical of those found in proactive individuals (Schjolden et al., 2005; Overli et al., 2007). These aggressive individuals also were able to compete successfully for resources within a population and hence typically attained dominant status in pairwise interactions with high responders (Schjolden et al., 2005; Overli et al., 2007). High responders, by contrast, demonstrated traits similar to those found in reactive individuals, and were likely to become subordinate in a social interaction (Schjolden et al., 2005; Overli et al., 2007). However, the behaviour for high and low responding lines was found to be context dependent and influenced by factors such as novel environments and group size (Schjolden et al., 2005; Schjolden et al., 2006).

Careau et al. (2008) suggested that stress-coping style could be related to energy metabolism and the metabolic rates of individuals. Generally, proactive animals are expected to adopt an energetically-expensive strategy, whereas reactive animals are expected to be energetically-conservative (Korte et al., 2005). The link between stress-coping style and RMR was supported by a recent study in the common carp (*Cyprinus carpio*) in which individuals that

exhibited risk-taking behaviour had higher metabolic rates and lower stress responsiveness, compared to individuals that demonstrated risk-avoidance behaviour (Huntingford et al., 2010). Similarly, in salmonids, individuals with higher metabolic rates were more likely to grow faster and become dominant in a social interaction than those with lower metabolic rates (Metcalfe and Thorpe, 1992; Metcalfe et al., 1995). Martins et al. (2011) also showed the existence of a relationship between stress-coping style (evaluated through escape behaviour and cortisol concentrations) and metabolic rate in Senegalese Sole (*Solea senegalensis*). This study showed that the extent of variation in oxygen consumption while in a respirometry chamber, which served as a novel environment, reflected the stress response of individuals. 'Active copers' (proactive individuals) demonstrated lower oxygen consumption rates 10 min after transfer to the chamber as well as during the 22 h measurement period, suggesting that these fish were quicker to recover from the stressor than passive or reactive individuals.

The evaluation of stress-coping style often relies on the measurement of cortisol response to a stressor (Overli et al., 2004; Schjolden et al., 2005; Overli et al., 2007; Ruiz-Gomez et al., 2008; Martins et al., 2011). Such measurements may be problematic in studies where the goal is to relate stress-coping style to other characteristics and therefore, behavioural indices of stress coping style have been sought. Several studies analyzing the behavioural differences between high and low responding lines of trout (Metcalfe and Thorpe, 1992; Overli et al., 2002) assessed stress-coping style through locomotor response to a territorial intrusion, the ability to attain dominance in a social interaction or the tendency to resume feeding after a stressor (Overli et al., 2007). The time taken to resume feeding after exposure to a stressor also has been used as an index of stress-coping style in studies on non-selected rainbow trout (Overli et al., 2002; Brelin et al., 2005; Overli et al., 2005; Overli et al., 2006). Proactive individuals (or low responders in

trout line studies) were usually observed to resume feeding more quickly after a stressor than reactive conspecifics (Overli et al., 2002; Overli et al., 2005; Overli et al., 2006; Overli et al., 2007). We explored the usefulness of this approach in zebrafish by measuring the time individuals took to resume feeding after exposure to a standardized stressor and comparing these data with whole-body cortisol responses to the same standardized stressor. We predicted that individuals that resumed feeding more quickly after a stressor (hence demonstrating a bolder, aggressive, proactive coping style) would have higher metabolic rates that their reactive conspecifics.

1.5.2b Relationships between aggression and metabolic rate

Aggression is one of the five broad axes of animal personality (Reale et al., 2007). This personality trait has been observed across a wide variety of contexts and situations such as in the acquisition and defense of resources, mates and territory. Prior studies on fish suggested that innate aggression contributes to the competitive ability of individuals which in turn could lead to dominance in social hierarchies (Riebli et al., 2011). Several studies assessed the consistency of aggressive behaviour over time. For example, McGhee and Travis (2010) found that dominance behaviours in bluefin killifish (*Lucania goodei*) remained consistent over time, while ayu (*Plecoglossus altivelis*) demonstrated consistent differences in their aggression levels directed at both model intruders and live conspecifics (Katano and Iguchi, 1996).

Experimentally, aggressive behaviour can be elicited through the use either of a conspecific intruder (as in many social hierarchy studies in salmonids; e.g. Metcalfe et al., 1989; Huntingford et al., 1990; McCarthy et al., 1992; Metcalfe et al., 1995; Sloman et al., 2000; Sloman et al., 2002) or a mirror. Mirror image stimulation (MIS) is a well-established method

that has been used to study behaviour in a variety of fish species (Tinbergen, 1951; Rowland, 1999; Gerlai et al., 2000; Marks et al., 2005; Castro et al., 2006; Moretz et al., 2007a; Moretz et al., 2007b; Desjardins and Fernald, 2010) because it provides immediate feedback on the subject's behaviour without the use of invasive procedures or excessive handling (Rowland, 1999). In MIS, an individual perceives an opponent that is matched in size, motivation and behaviour and this similarity increases the likelihood of escalation (Thompson, 1966; Gallup, 1968). For example, Tinbergen (1951) studied the effect of exposure to a mirror in three-spined stickleback (*Gasterosteus aculeatus*) and discovered that the fish treated their reflections as intruding conspecifics. Most recently, Desjardins and Fernald (2010) examined the effects on the brain and differences in aggression towards a mirror image or a live conspecific in *Betta splendens* males. When confronted with a mirror, fish had higher immediate early gene expression in brain areas homologous to the amygdala and hippocampus than when exposed to a live conspecific (Desjardins and Fernald, 2010). These results suggested that fish experienced fear when exposed to the mirror image, perhaps because they recognized something unusual in the behaviour or reactions of the mirror image. The effectiveness of MIS in eliciting aggressive behaviour in zebrafish has been reported in previous studies (e.g. Gerlai et al., 2000; Marks et al., 2005; Norton et al., 2011).

Recent work on animal personality has explored the impact that personality traits may have on an animal's daily energy expenditure (reviewed by Careau et al., 2008). In an attempt to determine the relationship between personality traits and metabolic rate, two competing models have been put forward: the performance model and the allocation model (Fig. 4. in Careau et al., 2008). The performance model posits a positive relationship between RMR and aggression or activity. It predicts that RMR reflects the size of the digestive or metabolic machinery needed to

capture, extract and mobilize energy. Hence, active individuals that sustain high levels of energy expenditure require larger-than-average organs that in turn require higher-than-average maintenance costs (Daan et al., 1990). In contrast, the allocation model predicts a negative relationship between RMR and the personality traits of aggression and activity. This model suggests that since animals have a limited energy supply, trade-offs have to occur between competing pathways. That is, energy allocated towards aggression and activity is lost from maintenance of RMR (Careau et al., 2008). In the present study, individual zebrafish were confronted with mirror images to provoke an aggressive response. We predicted that individuals that displayed higher levels of aggression and activity would also possess higher RMR, a prediction that is consistent with the performance model of (Careau et al., 2008).

1.6 Summary

In the last two decades, the zebrafish has emerged as a popular species for research in a variety of areas (Perry et al. 2010). The zebrafish is easily obtained, inexpensive to purchase and house, and easy to maintain in an aquatics facility, and therefore has become an important organism for research in physiology, endocrinology and toxicology. Zebrafish produce large numbers of transparent eggs that have proven useful in developmental, genetic and embryological studies. The zebrafish also has become an important tool for the study of human disease and biology, particularly in cardiovascular disease and cancer research (Perry et al. 2010). In the present study, we used this popular species to investigate factors that could account for intra-specific variation in metabolic rate.

CHAPTER TWO:

MATERIALS AND METHODS

2.1 Experimental Animals

Zebrafish (*Danio rerio*) were obtained from commercial suppliers (Big Al's, Ottawa, ON, Canada or AQuality, Mississauga, ON, Canada) and transported to the University of Ottawa Aquatic Facility where they were maintained in fibreglass tanks of either 3 L or 10 L volume. To reduce variation caused by potential differences between male and female fish, only female fish were used. Upon arrival at the Aquatic Facility, female fish were separated from male conspecifics. All tanks were maintained in a climate-controlled room (28 ± 1.5^{o}C) and were supplied with flowing, dechloraminated city of Ottawa tap water maintained at 28 ± 0.5^{o}C. The photoperiod was held constant at 14L:10D. Zebrafish were fed three times a day with a mix of 50% Adult Zebrafish Complete Diet (Zeigler Bros Inc., Gardeners, PA, USA), 25% Golden Pearls Shrimp Larval Diet (Artemia International, LLC, Camille, AZ, USA), and 25% Spirulina Aquarium flake food (Burlingame, CA, USA).

In experiments requiring identification of individual fish, a visible implant elastomer (Northwest Marine Technology Inc., Shaw Island, WA, USA) was used to give fish unique identification markers. Zebrafish were lightly anaesthetized (to the point of losing equilibrium) in an aerated, 28^{o}C solution of benzocaine (ethyl *p*-aminobenzoate; 1.25 µL mL^{-1}). The coloured polymer resin (red, yellow or green) was mixed with a curing agent in a 10:1 ratio (according to the manufacturer's instructions) and injected just under the skin on the dorsal surface of the fish using a 0.3 cc injection syringe. Zebrafish were allowed to recover from the procedure for a period of at least 5 days.

All animal holding and experimental protocols were approved by the University of Ottawa animal care committee (BL-229) and conformed to the guidelines of the Canadian Council on Animal Care for the use of animals in research and teaching.

2.2 Experimental protocols

The first step in all experiments was the measurement of resting metabolic rate (RMR) of individual zebrafish using intermittent closed-system respirometry. Respirometry measurements were carried out in parallel on four individual fish. Respirometry chambers were constructed from 20 mL plastic syringes (BD) supplied with flowing 28 ± 0.5°C water from an aeration column. The chambers were covered to avoid disturbance of the fish. Individuals were allowed to acclimate to the respirometry chambers overnight, and RMR measurements were initiated the next day at noon. For each fish, at least three measurements of oxygen consumption ($\dot{M}O_2$) were carried out, each approximately two hours apart, and the mean RMR was calculated. The rate of O_2 consumption in an empty chamber was also measured to account for background (bacterial) $\dot{M}O_2$. After completion of RMR measurements, individuals were removed from the respirometry chamber and lightly anaesthetized for the measurement of fork length and weight.

The rate of O_2 consumption was calculated from the decrease in the partial pressure of O_2 in the water (PwO_2) within the sealed respirometry chamber. To measure PwO_2, water was drawn from the chamber using a peristaltic pump (flow rate = 0.5 mL s^{-1}; Fisher Scientific, Ottawa, ON, Canada) and directed to a PO_2 electrode (Analytical Sensors E-101, Sugarland, Texas, USA) housed within a temperature-controlled cuvette, after which it was returned to the respirometry chamber. The PO_2 electrode was connected to a blood gas analyzer (Cameron Instruments BGM 200, Port Aransas, Texas, USA) linked to a data acquisition system (Biopac Systems Inc.) running AcqKnowledge data acquisition software on a PC. This system allowed continuous measurement of PwO_2. The PO_2 electrode was calibrated with solutions of sodium sulphite (20 mg ml^{-1}; PO_2 = 0 Torr) and air-saturated, dechloraminated city of Ottawa tap water.

Measurement of PwO_2 was carried out over a period of 20 min. For the initial 10 min, PwO_2 was measured while water flow to the respirometry chamber continued to ensure that fish were receiving air-equilibrated water. Water flow to the chamber was then stopped and PwO_2 was measured for a further 10 min. The rate of O_2 consumption was calculated from the fall in PwO_2 over the period in which chamber was sealed (initial water PO_2, PwO_{2i}, minus water PO_2 at the end of the closed period, PwO_{2f}), taking into account the volume of water in the chamber (vol_c), the solubility coefficient of O_2 in fresh water at 28°C ($\alpha_w O_2$; Boutilier et al. 1984), the time during which the chamber was sealed (t), and fish mass (where appropriate; see below); $\dot{M}O_2 = ((PwO_{2i} - PwO_{2f}) * \alpha_w O_2 * (vol_c/1000)) / (t/60)$.

2.2.1 Series 1: Repeatability of RMR

To assess the repeatability of RMR in individual zebrafish (n = 30; 0.45 ± 0.02 g; 3.7 ± 0.1 cm; values are means ± SEM), RMR was measured through intermittent closed-system respirometry, as described above, at two different times that were three weeks apart. Between trials, fish were returned to their 3 L holding tanks, where the feeding and care regime remained consistent for the experimental period. Following the second measurement of RMR, fish were terminally anaesthetized in a solution of benzocaine (ethyl *p*-aminobenzoate; 1g mL^{-1}) and sex was confirmed. Carcasses were stored at -80°C for later measurement of whole body cortisol concentrations (see below).

2.2.2 Series 2: The impact of baseline cortisol concentrations on RMR

To determine whether baseline cortisol concentrations contribute to individual variation in RMR, whole-body cortisol concentrations were measured for fish used in Series 1.

Measurement of whole-body cortisol concentrations was carried out as described by Fuzzen et al. (2010). In brief, zebrafish carcasses were thawed on ice, blotted dry and weighed. Fish were individually ground to a fine powder in liquid nitrogen using a mortar and pestle. Based on mass, samples were transferred into either one (<400 mg) or two (≥400 mg) 2 mL microtubes (Diamed Lab Supplies Inc., Mississauga, ON, CA) with 400 µL of homogenizing buffer (80 mM Na_2HPO_4; 20 mM NaH_2PO_4; 100 mM NaCl; 1 mM ethylenediaminetetraacetic acid) and homogenized (TH homogenizer, Omni International Inc., Marietta, GA, USA). Homogenates were extracted three times with 1 mL of ethanol each time. Extracted samples were held at 4°C for 60 min (30 min for last two extractions), centrifuged at 3000 *g* and 4°C, and frozen at -80°C for 10 min. All samples were subjected to overnight drying, under air, at room temperature. Samples were then reconstituted in 300 µL of acetate buffer, and passed through C_{18} solid phase extraction columns primed with 1 mL of methanol and 1 mL of double-distilled water. After addition of sample, 1 mL of ultra-pure water and 1 mL of hexane were added to the columns. Steroids were then eluted four times from the column using 1 mL of ethyl actetate (1% methanol) and were dried under air at room temperature. Finally, samples were reconstituted in 3 mL of assay buffer (21.4 mM $Na_2HPO_4 \cdot 7H_2O$; 9.3 mM $NaH_2PO_4 \cdot H_2O$; pH 7.6; 0.1% gelatin; 0.01% thimerosal). Using this approach, an extraction efficiency of 74 ± 1% was expected (Fuzzen et al., 2010).

Extracted and purified samples were stored at -80°C for later analysis of cortisol concentrations using a commercial EIA kit (Cayman Chemical, Ann Arbor, Michigan, USA) and SpectraMax® 340PC^{384} Absorbance Microplate reader (Molecular devices, LLC, Sunnyvale, CA, USA). Samples were assayed in duplicate. Intra-assay variation for measurements of baseline cortisol concentrations and post-stress cortisol concentrations, respectively, were 14.0 ±

1.7% and 9.5 ± 0.3%. In each case, all samples were measured in a single assay. Cortisol values were expressed per unit body mass.

2.2.3 Series 3: Is RMR related to stress-coping style?

To investigate possible relationships between RMR and stress-coping style, two responses to a standardized stressor were evaluated. Following measurement of RMR as described above, individual fish were placed in behavioural chambers for an overnight recovery and acclimation period. Fish were then subjected to a standardized net stressor (Ramsay et al., 2009), where individual fish were suspended in a net in air for 3 min, returned to their original holding chambers (but still kept within the net) for another 3 min, and then finally re-suspended in the air for 3 min. The time required to resume feeding post stress was then measured. Immediately after the stressor, a pinch of food was introduced into the tank, and the time until the first piece of food was taken was measured using a stopwatch. After a 24 h recovery period, fish were again subjected to the standardized stressor, and at 10 min post-stressor [chosen on the basis of the time course reported by Ramsay et al. (2009)], fish were euthanized by anaesthetic overdose for later measurement of whole body cortisol concentrations (as described above).

This experiment was carried out on three separate groups of fish, trial 1 (n=10; 0.45 ± 0.03 g; 3.5 ± 0.1 cm), trial 2 (n=12; 0.71 ± 0.03 g; 4.2 ± 0.1 cm) and trial 3 (n=11; 0.79 ± 0.04 g; 4.3 ± 0.1 cm). Data for trials 1 and 3 were combined for analysis. In the period preceding trial 2, husbandry procedures in the Aquatic Facility were changed such that fish were fed only twice a day as opposed to three times a day (the regime that was maintained for trials 1 and 3). This change in husbandry procedure may have affected the data collected in trial 2, which were therefore analyzed separately.

2.2.4 Series 4: Relationships between RMR and organ mass

To investigate relationships between RMR and organ mass, fish (n=39; 0.62 ± 0.03 g) were euthanized by anaesthetic overdose immediately following the measurement of RMR. Organs (heart, intestine with liver attached, and gills) were dissected out under a stereomicroscope (MZ6; Leica Microsystems Inc., Concord, ON, CA) and immediately weighed to the nearest 0.1 mg. The organs were then dried to constant mass in an oven at 60^{o}C and re-weighed. Pilot trials confirmed that 96 h at 60^{o}C was sufficient to achieve constant mass.

2.2.5 Series 5: Relationships between RMR and behaviour

Relationships between RMR and aggressive behaviour or activity were assessed using a single group of fish (n=35; 0.42 ± 0.07 g; 3.6 ± 0.04 cm). The procedure used was based upon that of Gerlai et al. (2000). Following the measurement of RMR, fish were transferred to individual behaviour chambers (15.1 cm x 9.3 cm x 15.0 cm) and allowed to recover/acclimate for 24 h. Each behaviour chamber was fitted with an external mirror angled at approximately 35^{o} to the plane of the transparent chamber wall; the other three chamber walls and the floor were opaque, while the lid of the tank was also opaque. The mirror was covered at the time the zebrafish was introduced into the tank. The experiment was initiated by recording the behaviour of the fish for 5 min (HC-V700M digital video camera, Panasonic, 60 frames s^{-1}); the camera was positioned directly over the tank. The cover was then removed from the mirror, and recording continued for an additional 10 min. All recordings were collected between 12 and 3 pm to minimize any confounding effects of circadian rhythms.

2.3 Data and statistical analyses

2.3.1 Residual analysis

Analysis of residuals was employed in Series 1: Repeatability of RMR and Series 4: Relationships between RMR and organ mass. Mass-independent $\dot{M}O_2$ (μmol h^{-1}) and body mass (g) were $\log_{10}$ transformed to linearize the relationship between these two variables prior to regression analysis using the least squares approach. The residual RMR (rRMR) was calculated as the difference between the observed and expected $\dot{M}O_2$ values for an individual of a particular mass from the relevant regression equation (as per Metcalfe et al., 1995). For the analysis of repeatability, residual RMR values calculated for week 2 were plotted against values calculated for week 1 to determine whether RMR was consistent across time (3 weeks) within an individual. Data collected for organ mass (g) and body mass (g) also were linearized (by $\log_{10}$ transformation) and residuals were calculated from the least squares linear regressions. Residuals calculated for mass-independent $\dot{M}O_2$ and organ mass were then plotted against each other to determine whether higher-than-expected $\dot{M}O_2$ could be attributed to larger-than-expected organ mass.

2.3.2 Quantification of activity and aggressive behaviours from video recordings

Video recordings of behaviour were analysed using VideoPoint software (Lenox Softworks Inc., Lenox, MA, USA). With this approach, an individual's location was tracked and marked within an x-y coordinate plane every 0.2 s; the x-y coordinate plane was calibrated across individuals by inputting the length of a consistent tank feature present in all trials. The final 2 min was used for analysis together with a 2 min period that occurred 5 min after exposure of the mirror. This time was chosen because preliminary analyses suggested that aggressive

behaviours peaked during this period. The digitized data points were then analysed using MatLab software (The MathWorks Inc., Natick, MA, USA) using a custom-written script designed to extract (1) the time spent in each of four equal quadrants within the tank, (2) the time spent in motion versus the time spent at rest, and (3) the total distance covered during the observation period. In addition, recordings were observed over the 10 min mirror exposure period and individuals were scored for the occurrence of specific behaviours. These behaviours included lunges or burst motion directed towards the mirror.

2.3.3 Statistical analysis

All statistical analyses were performed using SigmaPlot 11 (Systat Software, San Jose, CA) and a fiducial limit of significance of 0.05. Pearson's product-moment correlation (*r*) was used to analyze repeatability data (Series 1) and the relationship between organ mass and RMR (Series 4). Least squares regression analysis was used to determine whether a significant relationship existed between basal cortisol levels and RMR (Series 2). To evaluate relationships between RMR and stress-coping style (Series 3), mean values were calculated for individuals that fell into the 33^{rd} and 66^{th} percentile of responses to a stressor (time to resume feeding or cortisol response). Comparisons were then made between these groups using Student's *t*-tests. A similar approach was adopted to analyze data for aggressive behaviour and activity. Fish were ranked according to total distance moved during the control period and mean values were calculated and compared by Student's *t*-tests for individuals that fell into the 25^{th} and 75^{th} percentiles. Fish were also ranked independently according to number of aggressive acts directed at the mirror, changes in time spent in proximity to the mirror following its exposure, or changes in activity following exposure of the mirror. Mean values were calculated and

compared by Student's *t*-tests for individuals that fell into the 25^{th} and 75^{th} percentiles for each ranked data set.

CHAPTER THREE:

RESULTS

Zebrafish used in these experiments ranged in mass from 0.24 g to 0.99 g, averaging 0.45 ± 0.08 g (n=178) and in length from 3.0 cm to 4.6 cm, averaging 3.7 ± 0.03 cm (n=138). Absolute (untransformed) values for whole-animal RMR for these zebrafish ranged from 6.16 μmol h^{-1} to 13.10 μmol h^{-1}, averaging 9.05 ± 0.12 μmol h^{-1} (n=178).

3.1 Series 1: Repeatability of RMR

Resting metabolic rate was significantly related to body mass (Pearson product-moment correlation coefficient, r = 0.406, p = 0.026; data not shown), and therefore mass-independent RMR and body mass were $\log_{10}$ transformed to linearize the data prior to regression analysis (Fig. 3-1). Residuals for RMR were calculated for the initial and final RMR measurements (using the regression equations provided in Fig. 3-1) and plotted against each other. This analysis yielded a significant, positive relationship (Fig. 3-2; Pearson product-moment correlation coefficient, r = 0.425, p = 0.0192), indicating that individuals with higher-than-expected RMR retained this quality over a period of three weeks. Note that the strength of the relationship was improved to 0.538 (p = 0.003) by the removal of a single point (indicated by the arrow on Fig. 3-2).

3.2 Series 2: The impact of baseline cortisol concentrations on RMR

Measurement of whole-body cortisol concentrations for individuals used in the repeatability experiments of Series 1 did not reveal a significant relationship between basal cortisol concentrations and either mass-independent (Fig. 3-3; p = 0.275) or mass-dependent RMR (data not shown; p = 0.334).

Table 3-1. Mean length, mass and mass-independent resting metabolic rate ($\dot{M}O_2$) of zebrafish used in each experimental series.

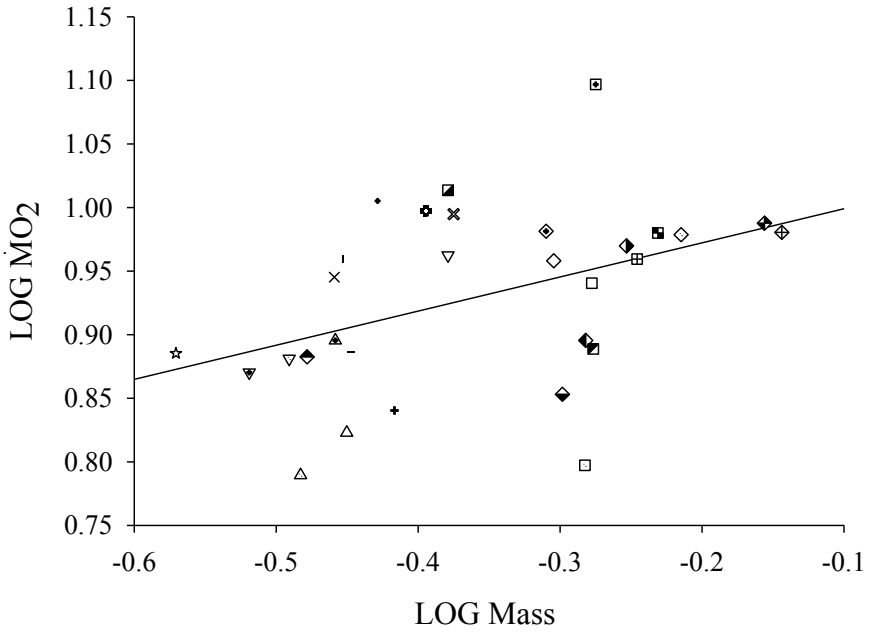
Experimental series	Length (cm)	Mass (g)	$\dot{M}O_2$ (μmol h^{-1})
Series 1: Repeatability (n = 30)	3.7 ± 0.1	0.45 ± 0.08	8.62 ± 0.26
Series 2: Baseline cortisol (n = 42)	3.6 ± 0.04	0.40 ± 0.01	8.31 ± 0.16
Series 3: Stress-coping style (trials 1 and 3 , no food restriction) (n = 20)	3.9 ± 0.1	0.62 ± 0.05	9.57 ± 0.40
Series 3: Stress-coping style study (trial 2, food restricted) (n = 12)	4.2 ± 0.1	0.71 ± 0.03	10.34 ± 0.39
Series 4: Organ mass (n = 39)	-*	0.62 ± 0.03	9.99 ± 0.23
Series 5: Personality (n = 35)	3.5 ± 0.04	0.42 ± 0.01	8.55 ± 0.23

Values are means ± SEM; n values are provided in parentheses for each experimental trial.

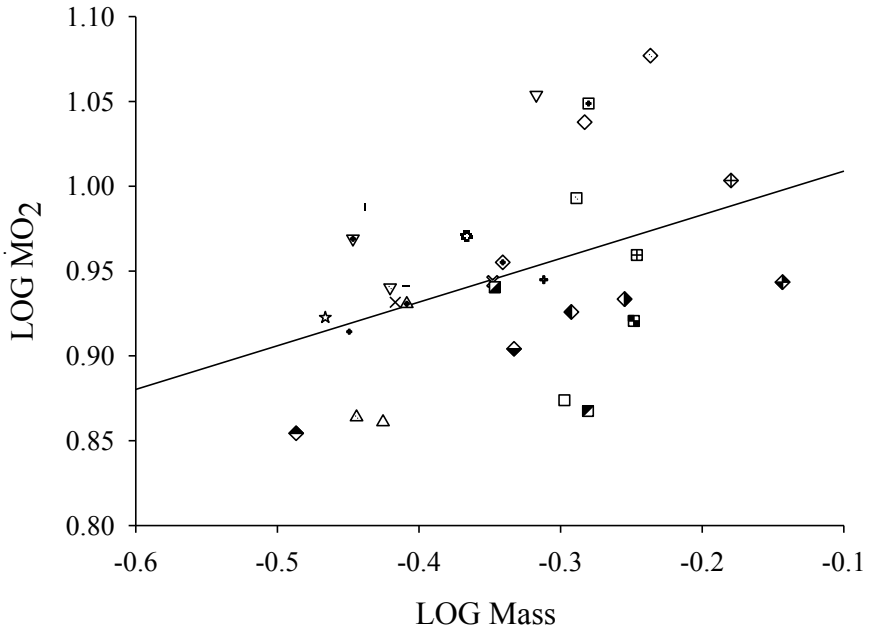
*Fish length data were not collected for this series of experiments.

Figure 3-1. Initial (A) and final (B; after three weeks) values for resting metabolic rate (RMR; measured as O_2 consumption by intermittent closed-system respirometry) and body mass of female zebrafish (*Danio rerio*) were plotted against each other after $\log_{10}$ transformation. Each fish (n = 30) is represented by a unique symbol. For (A), log $\dot{M}O_2$ = 0.2685 log mass + 1.026, p = 0.02, R^2 = 0.18; for (B), log $\dot{M}O_2$ = 0.2574 log mass + 1.035, p = 0.029, R^2 = 0.16.

A.


1.15
1.10
1.05
1.00
0.95
0.90
0.85
0.80
0.75
LOG $\dot{M}O_2$
-0.6
-0.5
-0.4
-0.3
-0.2
-0.1
LOG Mass

B.


1.10
1.05
1.00
0.95
0.90
0.85
0.80
LOG $\dot{M}O_2$
-0.6
-0.5
-0.4
-0.3
-0.2
-0.1
LOG Mass

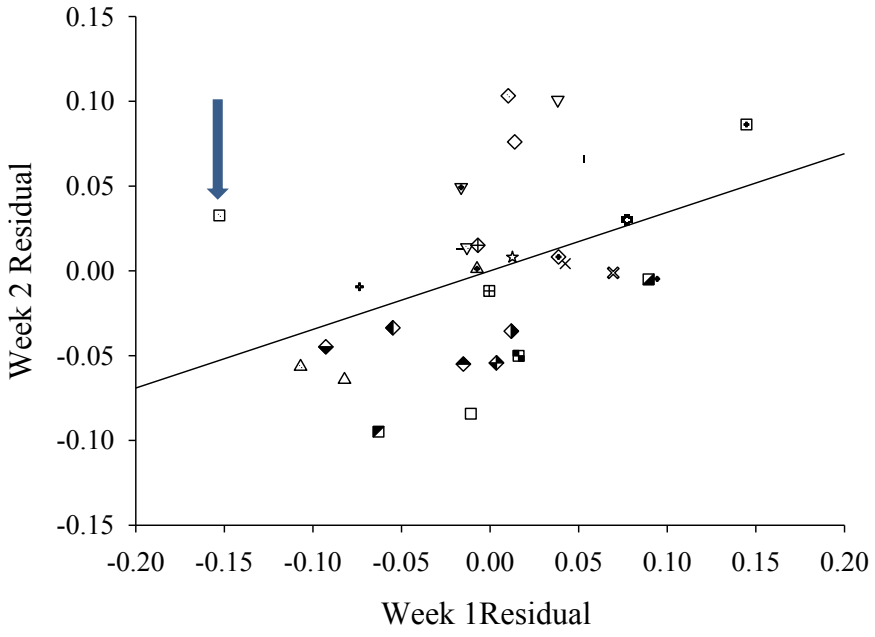


Figure 3-2. Residual values calculated from initial measurements of RMR (week 1; Fig. 3-1A) are plotted against residual values calculated for RMR measurements made three weeks later (week 2; Fig. 3-1B). Individual zebrafish (*Danio rerio*; n = 30) are identified with the same unique symbols used in Fig. 3-1. A significant positive relationship existed between initial and final residual RMR values (Pearson product moment correlation coefficient, Week 2 residual = 0.3455 Week 1 residual – (7.0×10^{-6}), r = 0.425, p = 0.0192). Note that the strength of the relationship was improved to 0.538 (p = 0.003) by the removal of a single point (indicated by the arrow on Fig. 3-2).

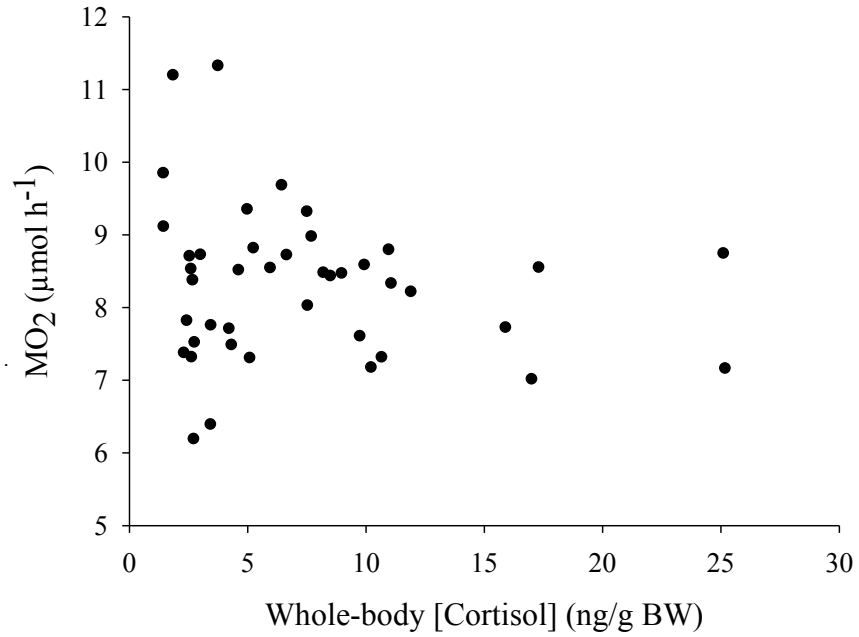


Figure 3-3. The relationship between resting metabolic rate (RMR; measured as O_2 consumption by intermittent closed-system respirometry) and baseline whole-body cortisol concentrations of female zebrafish (*Danio rerio*) was not significant (linear regression, $p = 0.275$). Each point represents an individual fish ($n = 42$).

3.3 Series 3: Is RMR related to stress-coping style?

Three separate trials were carried out to assess relationships between RMR and stress-coping style. During initial evaluation of results, marked differences were noted between trials 1 and 3, and trial 2. Specifically, fish in trial 2 resumed feeding after the stressor more rapidly (on average, 33.7 ± 8.4 s; n = 12) than fish in trials 1 and 3 (718.2 ± 233.0 s; n=21). Upon investigation it was discovered that feeding protocols in the Aquatic Facility changed over the course of this experimental series; in particular fish used in trial 2 were fed less often than those used in trials 1 and 3. Although there was no significant difference between RMR of fish in trials 1 and 3 (16.5 ± 1.0 μmol g^{-1} h^{-1}; n=21) versus trial 2 (14.9 ± 0.9 μmol g^{-1} h^{-1}; n=12), the data for trial 2 were analyzed separately from those of trials 1 and 3 to account for differences in time to resume feeding post-stress.

A standardized netting stressor (Ramsay et al. 2009) was used in these experiments. Comparison of whole-body cortisol concentrations post-stress (for fish of trials 1 and 3; n = 20) with baseline whole-body cortisol concentrations (see above) revealed that net-stressed individuals demonstrated significantly higher cortisol concentrations than non-stressed conspecifics (Fig. 3-4; Student's *t*-test, p = 0.037).

In addition to whole-body cortisol concentrations, time to resume feeding following the stressor was investigated as a possible indicator of stress-coping style. Time taken to resume feeding (for fish in trials 1 and 3; n = 20) was significantly related to post-stress whole-body cortisol concentration (Fig. 3-5; linear regression, p = 0.03, R^2 = 0.24). The relationship was positive, such that fish that exhibited higher whole-body cortisol concentrations post-stress also tended to take longer to resume feeding post-stress. However, the relationship was driven by two fish that exhibited unusually long times to resume feeding together with high post-stress whole-

body cortisol concentrations, and overall, post-stress cortisol concentration explained only 24% of the variation in time to resume feeding, suggesting that the relationship was not particularly robust. Increasing the number of observations would help to determine whether time to resume feeding is a useful index of stress-coping style in zebrafish.

To investigate possible relationships between RMR and stress-coping style, fish (of trials 1 and 3) were ranked according to either whole-body cortisol concentrations post-stress, or time to resume feeding post-stress. Individuals that ranked above the 33rd percentile (i.e. lowest cortisol concentrations or shortest time to resume feeding post stress) were deemed to be proactive (n = 7) whereas fish that ranked below the 66th percentile (i.e. highest cortisol concentrations or longest times to resume feeding post stress) were considered to be reactive (n = 7). Although in each case these groups differed significantly in the variable on which they were ranked ([cortisol] post-stress, Student's *t*-test, $p = 0.007$; time to resume feeding post-stress, Student's *t*-test. $p < 0.001$), no significant differences in RMR were detected between proactive and reactive fish (Fig. 3-6).

Interestingly, in the fish of trial 2, which experienced a somewhat restricted feeding regime relative to those of trials 1 and 3, a significant relationship was detected between RMR and time to resume feeding post-stress (Fig. 3-7; linear regression, $p = 0.018$, $R^2 = 0.44$). Note that regression analysis was used rather than dividing the fish into proactive and reactive individuals because of the low number of fish in this group (n = 12).

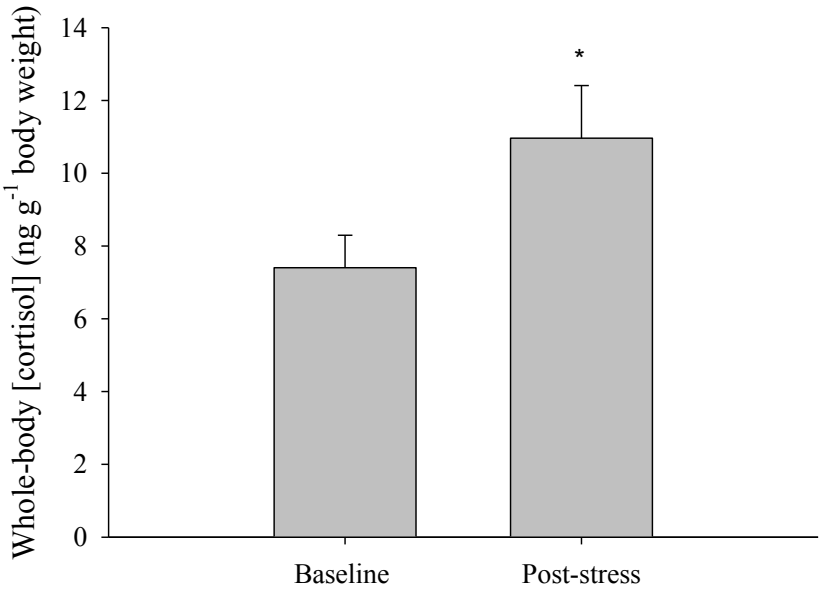


Figure 3-4. Whole-body cortisol concentrations of female zebrafish (*Danio rerio*) under baseline conditions (n = 42) or following a standardized netting stressor (n = 20). Values are means ± SEM. The asterisk denotes a significant difference between the two groups (Student's *t*-test, p = 0.037).

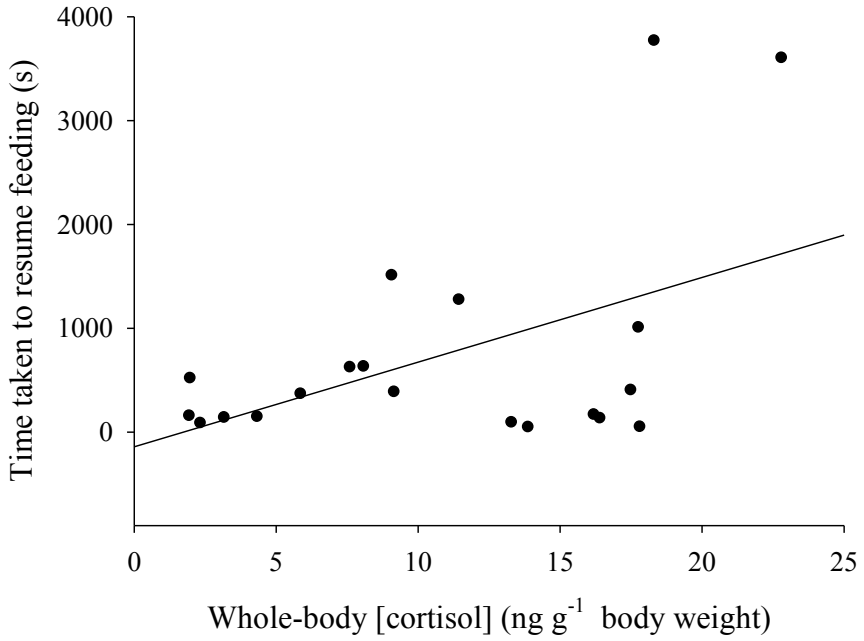
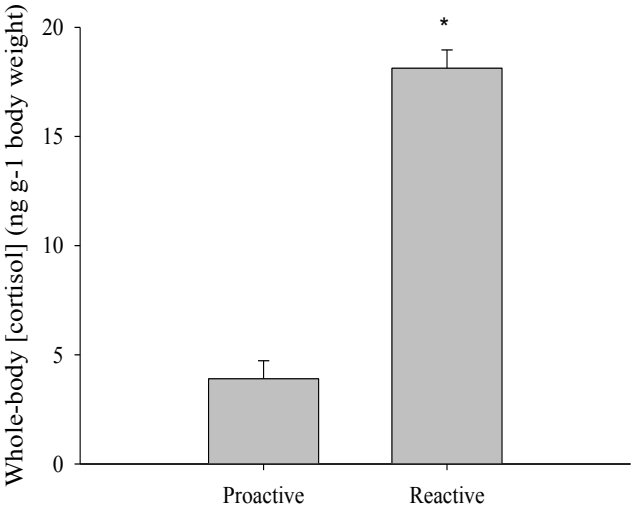


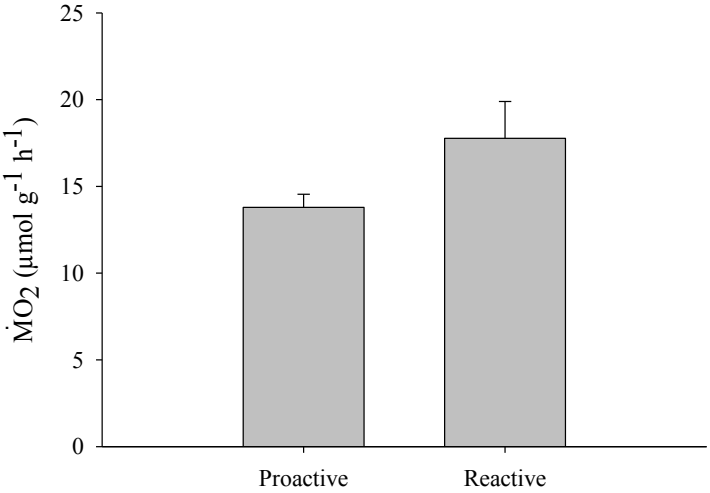
Figure 3-5. The relationship between time taken to resume feeding post-stress and whole-body cortisol concentration post-stress in female zebrafish (*Danio rerio*) used in trials 1 and 3 of stress-coping style experiments (Series 3). The relationship was described by the equation time to resume feeding = 81.539 [cortisol] – 141.61, least squares linear regression, $p = 0.03$, $R^2 = 0.24$. Each point represents data collected from a single fish (n = 20).

Figure 3-6. Zebrafish (*Danio rerio*) were identified as exhibiting a proactive or reactive stress-coping style on the basis of (A,B) the whole-body cortisol response to a standardized netting stressor or (C,D) the time taken to resume feeding following the standardized netting stressor. Panels A and C present, respectively, mean post-stress whole-body cortisol concentrations and mean time to resume feeding post-stress for fish ranked above the 33rd percentile (deemed to be proactive) or below the 66th percentile (deemed to be reactive). Panels B and D present data for resting metabolic rate (RMR; measured as O_2 consumption by intermittent closed-system respirometry) for the resultant groups of proactive and reactive fish. A significant difference between the groups is indicated with an asterisk (Student's *t*-test, p = 0.007 for panel A, 0.597 for panel B, <0.001 for panel C and 0.090 for panel D). Data are means ± SEM (n = 7 in all cases).

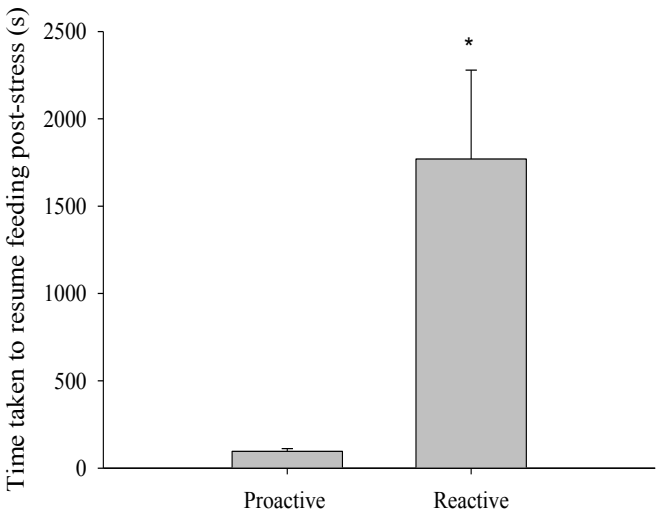
A.


Whole-body [cortisol] (ng g-1 body weight)
20
15
10
5
0
*
Proactive
Reactive

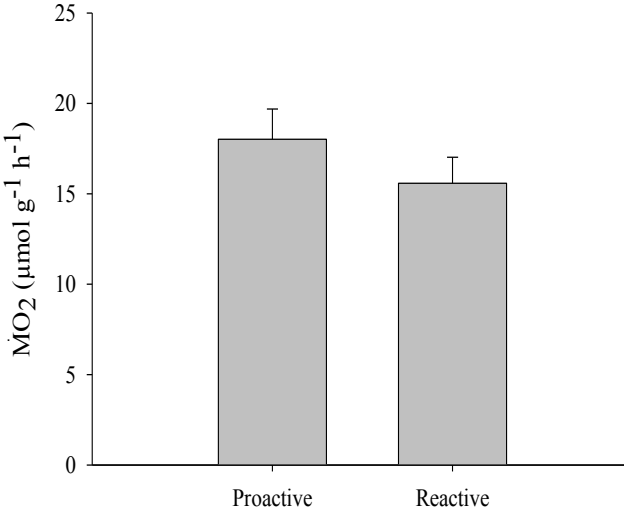
B.


$\dot{M}O_2$ (µmol g^{-1} h^{-1})
25
20
15
10
5
0
Proactive
Reactive

C.


Time taken to resume feeding post-stress (s)
2500
2000
1500
1000
500
0
*
Proactive
Reactive

D.


$\dot{M}O_2$ (µmol g^{-1} h^{-1})
25
20
15
10
5
0
Proactive
Reactive

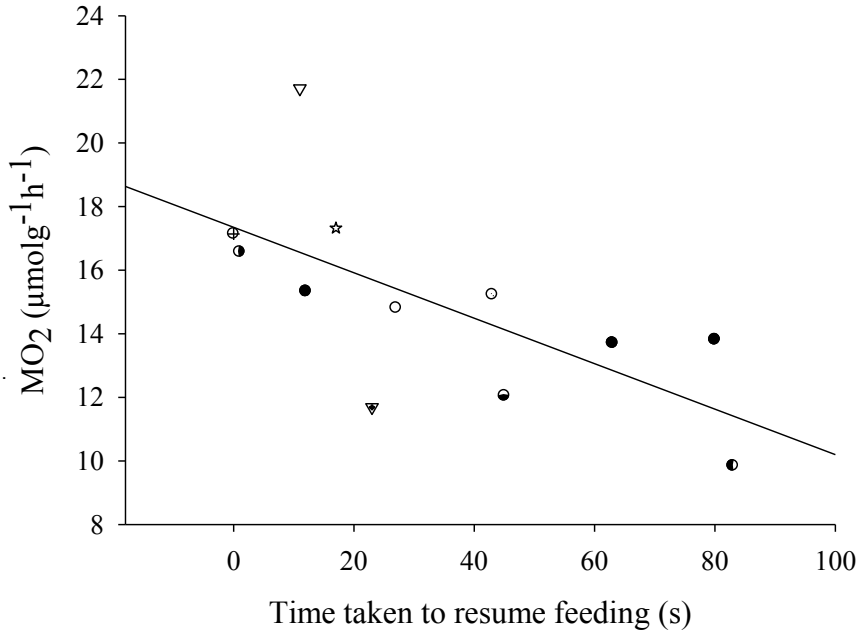


Figure 3-7. A significant relationship was detected between resting metabolic rate (RMR; measured as O_2 consumption by intermittent closed-system respirometry) and time taken to resume feeding following a standardized netting stressor for female zebrafish (*Danio rerio*) that experienced a somewhat restricted feeding regime during the experiment (linear regression, $\dot{M}O_2$ = -0.0715 time to resume feeding + 17.348, $p = 0.018$, $R^2 = 0.44$). Each data point represents a single fish (n = 12).

3.4 Series 4: Relationships between RMR and organ mass

All data (Table 3-2) were $\log_{10}$ transformed for analysis, as in prior similar studies (e.g. McCarthy, 2000; Maciak and Konarzewski, 2010; Norin and Malte, 2012). In all cases, organ wet weights were significantly related to body mass (Table 3-2). Gill dry mass, and intestine plus liver dry mass also exhibited significant positive correlations with body mass (Table 3-2); heart dry mass was below the limit of reliable measurement. To determine whether individuals with higher-than-expected active organ masses also possessed relatively high RMR, residuals calculated for RMR and organ masses (Table 3-3) were plotted against each other. No significant relationship was found between residual RMR and residuals for either the wet or dry mass of any organ examined (Table 3-4). In addition, no significant correlation was found between the residual of the sum of all organ masses and the residual of RMR.

3.5 Series 5: Relationships between RMR and behaviour

Relationships between RMR and two aspects of behaviour, activity and aggression, were explored. Indices of routine activity were determined from the 2-min control video recordings of zebrafish that were acquired after the 24-h acclimation period to the experimental chamber and immediately before the exposure of the mirror. Total distance moved during the 2 min control period ranged from 0.3 to 1116.8 cm, averaging 534.9 ± 46.2 cm (n = 35) and was not significantly correlated with body mass (Pearson product moment correlation coefficient, r = 0.183, p = 0.293). Therefore, fish were ranked according to total distance moved during the 2-min control period and mass-dependent RMR was compared between fish that moved the most (top 25th percentile) and least (fish ranked below the 75th percentile). Although these groups differed significantly in the total distance moved during the 2 min observation period (Student's

t-test, p <0.001) no significant difference in mass-dependent RMR was detected, although a trend was apparent for fish that moved less to exhibit higher mass-dependent RMR (Student's *t*-test, p = 0.059; Fig. 3-8). Resting metabolic rate was also compared between fish that spent 0% of their time at rest (n = 20) and those that spent 19.4% to 99.5% of their time at rest (n = 7). However, no significant difference in RMR was found between these two groups (20.3 ± 1.0 μmol g^{-1} h^{-1} for fish that spent 0% of time at rest versus 20.3 ± 1.4 μmol g^{-1} h^{-1} for fish that spent at least 19.4% of time at rest; Student's *t*-test, p = 0.997).

Several indices of aggressive behaviour were investigated. First, fish were scored for the number of aggressive acts (e.g. lunges or bursts of speed directed towards the mirror) carried out towards the mirror image during the 10 min period of exposure. The number of aggressive acts ranged from 0 to 47, averaging 14.4 ± 2.2 (n = 35). Fish were then ranked according to the number of aggressive attacks and mass-dependent RMR was compared between fish that were most (top 25th percentile) and least aggressive (fish ranked below the 75th percentile). Although these groups differed significantly in the number of aggressive acts (Student's *t*-test, p < 0.001), no significant difference in mass-dependent RMR was detected (Student's *t*-test, p = 0.499; Fig. 3-9).

Time spent in proximity to the mirror image was used as a second index as of aggressive behaviour. During the control period, fish spent on average 39.9 ± 4.3% (n=35) of the observation period in the half of the tank where the (covered) mirror was located, a value that was slightly but significantly (one-sample Student's *t*-test, p =0.029) lower than the value of 50% that would be expected if fish were making equal use of all tank area. During the observation period 5 min after the mirror was uncovered, average time spent in the half of the tank next to the mirror decreased significantly (paired Student's *t*-test, p = 0.013) to 33.1 ± 3.6%

(n = 35) of the observation period. Despite this overall trend, individual fish varied in their response to the mirror, with the change in percent of time spent in the half of the tank next to the mirror ranging from -79.7% (i.e. less time next to the exposed mirror) to 26.9% (i.e. time spent next to the mirror increased when the mirror was exposed). Fish were ranked on the basis of this change in percent time spent in the half of the tank next to the mirror, and mass-specific RMR was compared between fish exhibiting the greatest decrease (fish deemed to be less aggressive) and greatest increase (fish deemed to be more aggressive) in time spent next to the mirror when the mirror was exposed. Although these groups differed significantly in their response to the mirror (Student's *t*-test, $p < 0.001$), no significant difference in mass-specific RMR was detected (Student's *t*-test, $p = 0.759$; Fig. 3-10).

Total distance moved in the absence/presence of the mirror image was used as a final index of aggressive behaviour. During the control observation period, fish moved on average 534.9 ± 46.2 cm (n = 35). Five minutes after exposure to the mirror, total distance moved during the observation period had decreased significantly (paired Student's *t*-test, $p = 0.025$) by 36.1 ± 27.2 cm (n = 35) to 498.8 ± 37.0 cm (n = 35). This average, however, masked considerable variation among individuals in the change in activity that occurred upon exposure of the mirror, with individual responses ranging from a 383.9 cm decrease in total movement to a 623.0 cm increase in total movement following exposure of the mirror. Thus, fish were ranked on the basis of the change in total movement following exposure of the mirror, and mass-specific RMR was compared between fish exhibiting the greatest decrease (fish deemed to be less aggressive) and greatest increase (fish deemed to be more aggressive) in total distance moved following exposure of the mirror. Although these groups differed significantly in their response to the mirror

(Student's *t*-test, $p < 0.001$), no significant difference in mass-specific RMR was detected (Student's *t*-test, $p = 0.926$; Fig. 3-11).

Table 3-2. Mean, minimum and maximum organ masses of 39 female zebrafish (*Danio rerio*) together with statistical analysis of the relationship between organ mass and whole-body resting metabolic rate (RMR; measured as O_2 consumption by intermittent closed-system respirometry).

Organs (Wet/Dry)	*Mass (mg)*			*Pearson product-moment correlation coefficient*	
	Mean ± SEM	*Minimum*	*Maximum*	*R*	*p*
Heart (Wet)	1.9 ± 0.1	0.3	3.1	0.392	0.0135
Gills (Wet)	30 ± 1.1	10.0	40.0	0.766	<0.001
Intestine and Liver (Wet)	60 ± 2.4	30.0	100.0	0.833	<0.001
Gills (Dry)	3.7 ± 0.4	1.2	15.5	0.609	<0.001
Intestine and Liver (Dry)	9.9 ± 0.54	4.2	18.3	0.604	<0.001

Table 3-3 Equations of least-squares regression lines used to calculate the residuals for $\log_{10}$ resting metabolic rate (RMR) or the $\log_{10}$ masses of the different organs studied with respect to $\log_{10}$ body mass.

Variable (y)	*Linear squares regression equation*	R^2	*p*
RMR	y = 0.3093x + 1.065	0.377	<0.001
Heart (Wet)	y = 0.6151x – 2.624.	0.131	0.014
Gills (Wet)	y = 0.7525x – 1.429	0.576	<0.001
Gut and Liver (Wet)	y = 0.7098x – 1.08	0.686	<0.001
Gills (Dry)	y = 1.0884x – 2.249	0.354	<0.001
Gut and Liver (Dry)	y = 0.7191x – 1.865	0.348	<0.001

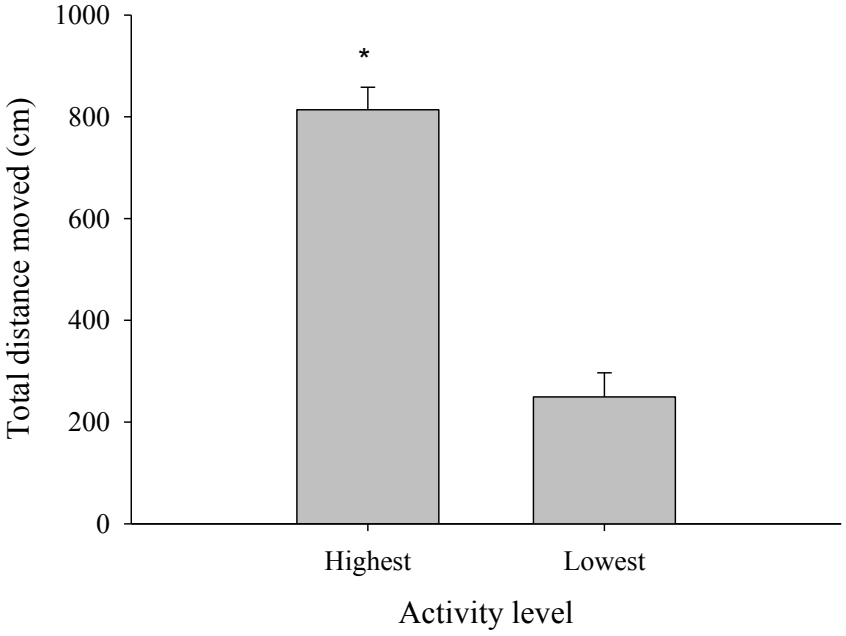
x represents $\log_{10}$ body mass.

Table 3-4. Pearson product moment correlation coefficients (r) for residual $\log_{10}$ resting metabolic rate (RMR; measured as O_2 consumption by intermittent closed-system respirometry) as a function of residual $\log_{10}$ organ wet or dry mass for 39 zebrafish (*Danio rerio*).

Organs (Wet/Dry mass)	*p value*	*Correlation coefficient (r)*
Heart (Wet)	0.541	0.101
Gut and Liver (Wet)	0.125	0.250
Gills (Wet)	0.909	-0.0190
Gut and Liver (Dry)	0.307	0.168
Gills (Dry)	0.869	0.0272
Cumulative wet weight	0.256	0.186
Cumulative dry weight	0.392	-0.141

Figure 3-8 Mean total distance travelled during the control observation period and (B) mean mass-specific resting metabolic rate (RMR; measured as O_2 consumption by intermittent closed-system respirometry) for female zebrafish (*Danio rerio*) divided into groups that exhibited the lowest and highest activity levels. An asterisk indicates a significant difference between the groups (Student's *t*-test, $p < 0.001$ for panel A and 0.059 for panel B). Values are means ± SEM, with n = 12 (both low and high activity groups).

A.


1000
800
600
400
200
0
Total distance moved (cm)
*
Highest
Lowest
Activity level

B.

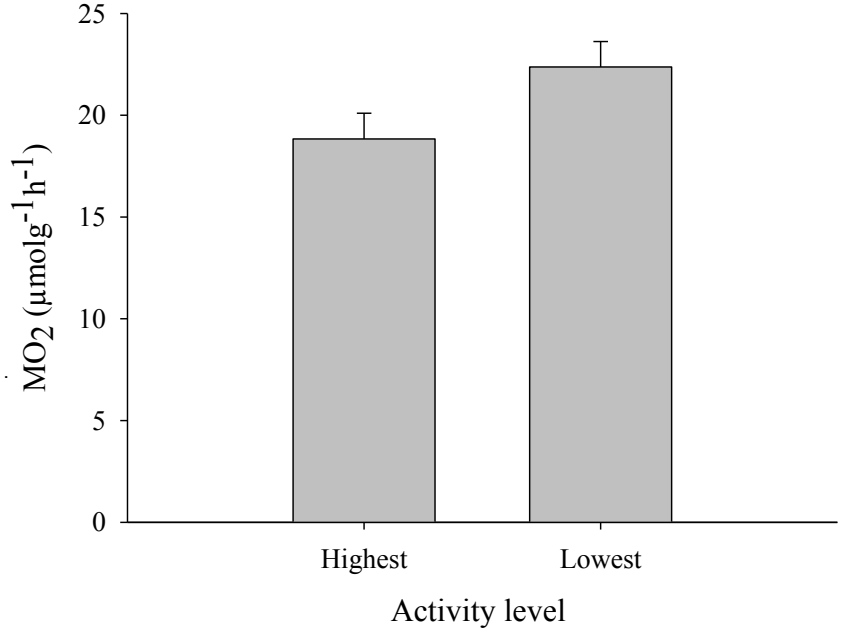
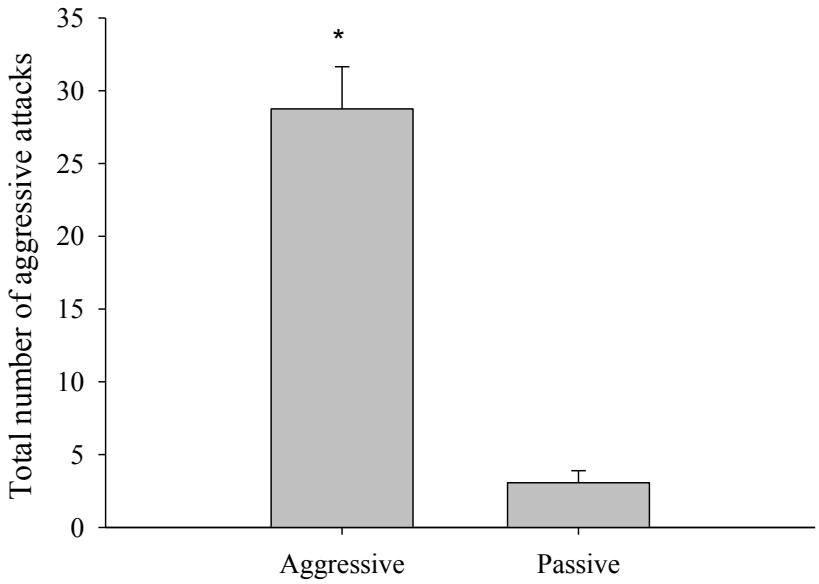

25
20
15
10
5
0
$\dot{M}O_2$ (μmolg^{-1}h^{-1})
Highest
Lowest
Activity level

Figure 3-9. (A) Mean number of attacks directed towards a mirror image and (B) mean mass-specific resting metabolic rate (RMR; measured as the rate of O_2 consumption by intermittent closed-system respirometry) for female zebrafish (*Danio rerio*) divided into groups deemed to contain aggressive and passive individuals (see text). An asterisk indicates a significant difference (Student's *t*-test, $p < 0.001$ for panel A and 0.499 for panel B). Values are means ± SEM; aggressive fish, n = 12; passive fish, n = 14.

A.


Total number of aggressive attacks
35
30
25
20
15
10
5
0
*
Aggressive
Passive

B.

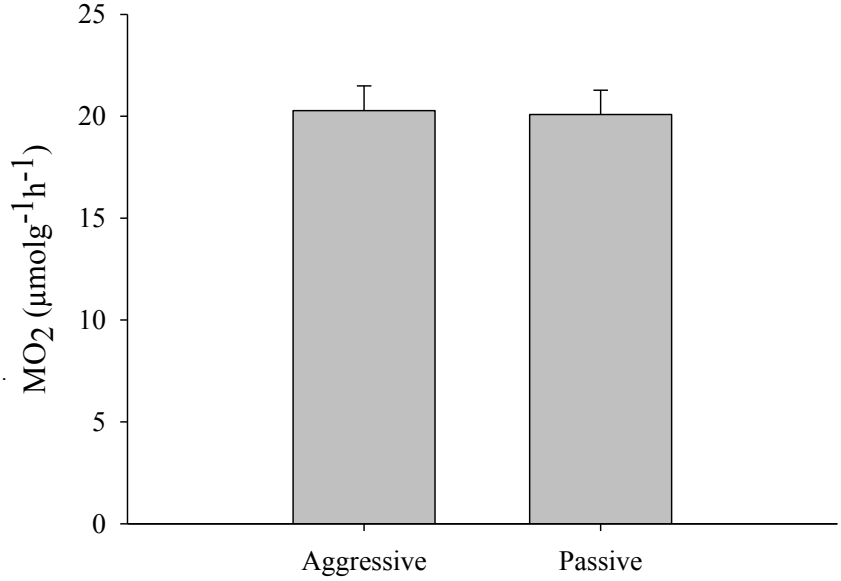
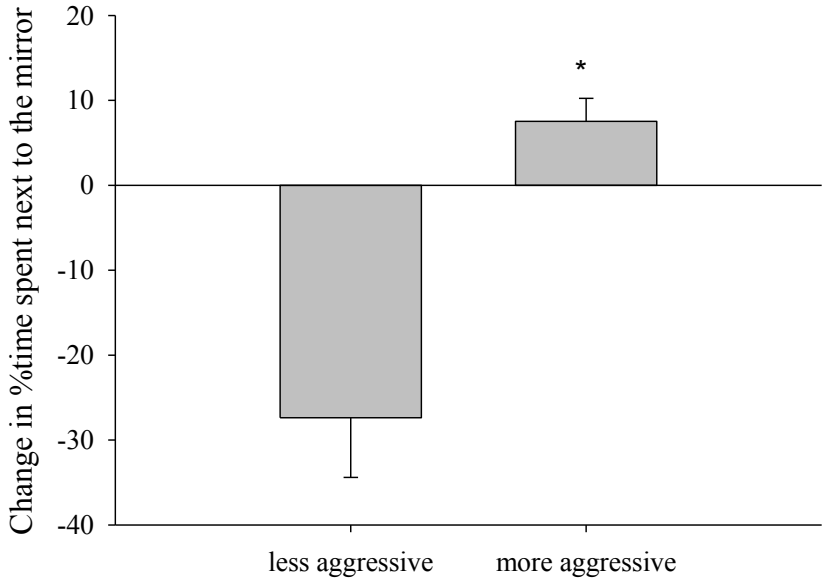

$\dot{M}O_2$ (µmolg^{-1}h^{-1})
25
20
15
10
5
0
Aggressive
Passive

Figure 3-10. (A) Mean change in percent time spent next to the mirror following exposure of the mirror and (B) mean mass-specific resting metabolic rate (RMR; measured as the rate of O_2 consumption by intermittent closed-system respirometry) for female zebrafish (*Danio rerio*) divided into less and more aggressive groups (see text). An asterisk indicates a significant difference between the groups (Student's *t*-test, $p < 0.001$ for panel A, 0.759 for panel B). Values are means ± SEM, n = 9 (for both more and less aggressive groups).

A.


Change in %time spent next to the mirror
20
10
0
-10
-20
-30
-40
*
less aggressive
more aggressive

B.

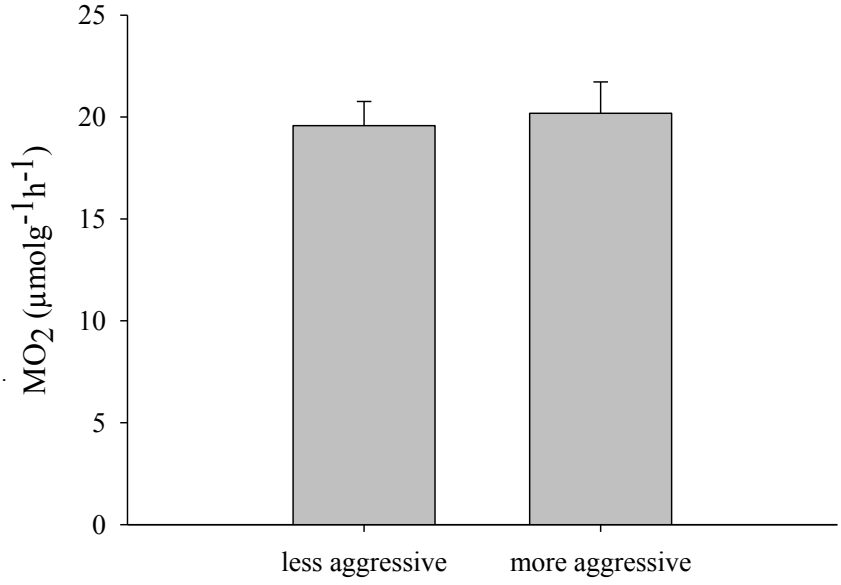
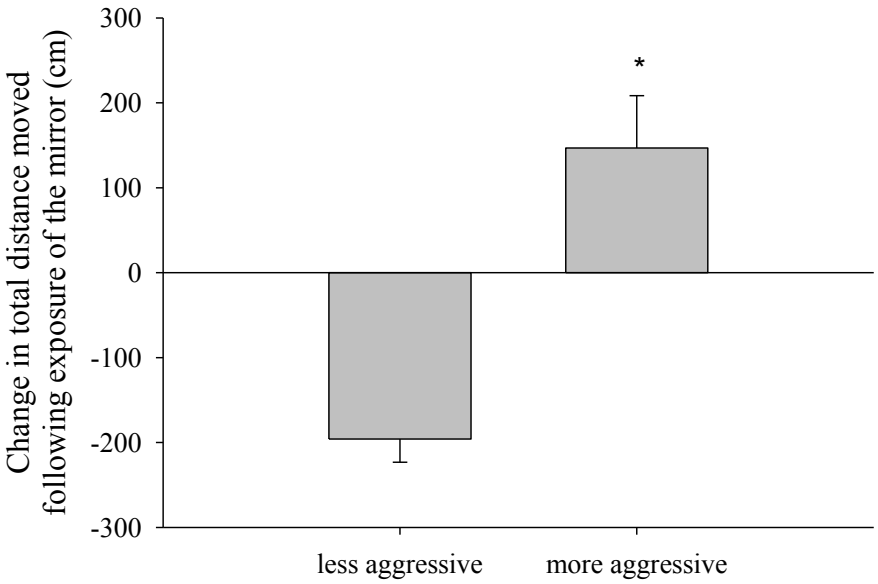
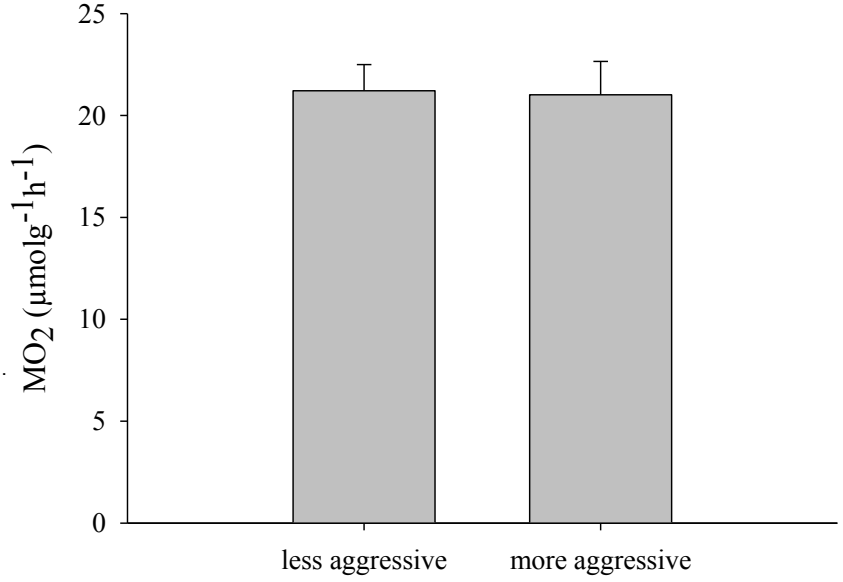

$\dot{M}O_2$ (µmolg^{-1}h^{-1})
25
20
15
10
5
0
less aggressive
more aggressive

Figure 3-11. (A) Mean change in total distance moved upon exposure of the mirror and (B) mean mass-specific resting metabolic rate (RMR; measured as the rate of O_2 consumption by intermittent closed-system respirometry) for female zebrafish (*Danio rerio*) divided into groups of less and more aggressive individuals (see text). An asterisk indicates a significant difference between the groups (Student's *t*-test, $p < 0.001$ for panel A, 0.926 for panel B). Values are means ± SEM, n = 9 (for both more and less aggressive group).

A.


Change in total distance moved following exposure of the mirror (cm)
300
200
100
0
-100
-200
-300
*
less aggressive
more aggressive

B.


$\dot{M}O_2$ ($\mu molg^{-1}h^{-1}$)
25
20
15
10
5
0
less aggressive
more aggressive

CHAPTER FOUR:

DISCUSSION

Variation in RMR across species largely reflects variation in body mass, with factors such as climate, water accessibility, temperature and diet playing minor roles (De Boeck et al., 2001; Careau et al., 2008). As in a number of other studies of intra-specific variation in RMR (see Careau et al., 2008), whole-animal RMR in zebrafish exhibited a significant but weak dependence on body mass. The low percentage of variance in RMR explained by body mass (e.g. 17-27% in the present study) is thought to reflect the influence of other factors on RMR. However, identifying the factors that account for this variation in RMR among individuals within a species has proven to be difficult (Careau et al., 2008; Burton et al., 2011; Konarzewski and Ksiazek, 2013). The results of the present study provide a case in point – despite considerable variation in RMR among the individual zebrafish used over the course of the present study, RMR was not significantly correlated with any of the factors investigated as possible drivers of inter-individual variation in RMR. In the discussion below, methodological issues surrounding the measurement of RMR and the repeatability of these measurements are considered before turning to the factors investigated as potential explanations of individual variation in RMR.

4.1 Measurement of RMR

The present study used intermittent closed-system respirometry to determine the rate of O_2 consumption as a measure of RMR. This approach is a variation on closed system respirometry in which relatively short periods of closed system respirometry are interspersed with flushing of the respirometry chamber, thereby avoiding problems of CO_2 accumulation (Steffensen, 1989). Ideally, the rate of decline of PO_2 is measured during each closed period (Steffensen, 1989), but artifacts on the PO_2 trace associated with opening or closing water flow valves in the present study required that ΔPO_2 (i.e. initial PO_2 – final PO_2) be used in the

calculation of $\dot{M}O_2$ rather than the rate of decline of PO_2 over the respirometry period. To minimize variation introduced by differences in final PO_2 on a small ΔPO_2 values, PO_2 differences of 15-20 Torr were employed (requiring that the respirometer be closed for 10 min). Zebrafish were acclimated to the respirometry chamber overnight to minimize stress-induced elevations of $\dot{M}O_2$ that typically accompany introduction of the animal into a novel environment (e.g. Martins et al., 2011), and $\dot{M}O_2$ measurements for all fish were carried out at the same time of day to minimize the impact of circadian variation in metabolic rate (Steffensen, 1989; Ros et al., 2004). Collectively, these approaches served to minimize measurement error and variation caused by external factors so as to emphasize the contribution of biological variation, i.e. attributes of the individual being measured, to inter-individual differences in RMR (see Careau et al., 2008).

4.2 Repeatability of RMR

The repeatability of RMR was evaluated to determine the extent to which a single measurement of RMR is reflective of future measurements and performance. Resting metabolic rate in zebrafish was repeatable over a period of three weeks. Similarly, mass-corrected SMR in spined loach was found to be repeatable over a period of 5 months (Maciak and Konarzewski, 2010), metabolic rate in Atlantic salmon showed consistency over 113 days (McCarthy, 2000), and SMR (along with active metabolic rate and absolute aerobic scope) in brown trout was significantly repeatable over 5 weeks (Norin and Malte, 2011). However, in both Atlantic salmon (McCarthy, 2000) and brown trout (Norin and Malte, 2011), repeatability of metabolic rate declined with longer periods of time, disappearing after several months. A recent meta-analysis of studies on both ectothermic and endothermic animals also reported a decline in the

repeatability of metabolic rate as the interval between measurements increased (White et al., 2013). Although the reasons for a decline in the repeatability of metabolic rate over time remain largely unexplored, plasticity of metabolic pathways has been suggested as a possibility, at least in cases where food availability varied (O'Connor et al., 2000; Norin and Malte, 2011). For example, O'Connor et al. (2000) reported that relative SMR of juvenile Atlantic salmon was stable over a three month period when food was not limiting, but decreased when fish were deprived of food. Similarly, Norin and Malte (2011) attributed the decline in repeatability of metabolic rate over time that they observed in brown trout to the use of a restricted food regime, which could both lower metabolic rate in and of itself, but also could diminish potential benefits associated with high SMR leading to changes in metabolic rate over time. In the present study, the repeatability of RMR probably reflected both the relatively short time interval between measurements (21 days, chosen to check whether individuals retained their RMR ranking over the course of an experimental series, which was typically carried out over a period of three weeks), and the fact that zebrafish were maintained under conditions of high food availability.

4.3 Relationships between baseline cortisol concentrations and RMR

The glucocorticoid hormone cortisol is well-known as a stress hormone in fish, as in other vertebrates (reviewed by Wendelaar Bonga, 1997; Mommsen et al., 1999). The main targets for cortisol in fish include the gill, intestine and liver, reflecting the two major actions of this hormone, i.e. regulation of salt and water balance, and regulation of energy metabolism (Wendelaar Bonga, 1997; Mommsen et al., 1999). Although many studies have focused on the cortisol response to a stressor and the subsequent effects of cortisol on carbohydrate, protein and lipid metabolism (reviewed by Mommsen et al., 1999), few have considered the influence of

variation in baseline cortisol levels on any aspect of physiology in fish. Recent interest in potential links between glucocorticoid concentrations and fitness has highlighted the paucity of studies that have focused on variation in baseline cortisol levels (Bonier et al., 2009). Given the well-documented effects of cortisol on intermediary metabolism (Mommsen et al., 1999), a causal relationship between baseline cortisol concentrations and RMR is a plausible hypothesis. Moreover, experimental elevation of circulating cortisol levels increased $\dot{M}O_2$ in both rainbow trout (De Boeck et al., 2001) and cutthroat trout (*Oncorhynchus clarki clarki*) (Morgan and Iwama, 1996). Nevertheless, no significant relationship was found between baseline cortisol levels and RMR in zebrafish of the present study. This result suggests that individual differences in the 'housekeeping' concentrations of cortisol are insufficient to generate measurable differences in whole-animal RMR. Cortisol is not, however, the only endocrine regulator of metabolism. For example, RMR was correlated with circulating levels of 11-ketotestosterone in male Mozambique tilapia (*Oreochromis mossambicus*), and 11-ketotestosterone treatment was associated with a significant elevation of RMR (Ros et al., 2004). Thus, future studies on the causes of intra-specific variation in RMR in zebrafish should consider investigation of a broader range of hormones, particularly reproductive hormones.

4.4 Relationships among stress-coping style, behaviour and RMR

To distinguish between proactive and reactive zebrafish, the whole-body cortisol response to a standardized net stressor (Ramsay et al., 2009) was examined. As reported by Ramsay et al. (2009), exposure to the net stressor resulted in a significant elevation of whole-body cortisol concentrations. However, post-stress cortisol concentrations measured in the present study (11 ng g^{-1} fish) were considerably lower than those reported previously (~28 ng g^{-1}

fish; Ramsay et al., 2009). The use of different cortisol extraction procedures between the two studies may account for at least part of this difference. The extraction protocol used in the present study was based on that of Fuzzen et al. (2010). Using a different stressor (vortexing), Fuzzen et al. (2010) reported whole-body cortisol concentrations in zebrafish of 20-25 ng g^{-1} fish post-stress, also considerably higher than those measured in the current study. However, both Ramsay et al. (2009) and Fuzzen et al. (2010) used a mix of male and female zebrafish, whereas only female zebrafish were used in the present study. A recent study reported a significant degree of sexual dimorphism in the stress response of zebrafish, with female fish exhibiting significantly lower stress-induced levels of cortisol than male fish (Mueller and Diamond, 2001). Despite the overall low cortisol response, substantial individual variation in post-stress whole-body cortisol concentrations was observed enabling low-responding or proactive fish to be distinguished from high-responding or reactive individuals.

Time to resume feeding post stress was investigated as a second possible indicator of stress-coping style in zebrafish. In rainbow trout lines selected for divergent cortisol responses to a standardized stressor, 'high responders', i.e. fish selected for a larger cortisol response to a standardized stressor, were significantly less likely to feed following transfer to a new environment than were 'low responders'(Overli et al., 2002). Similarly, rainbow trout that resumed feeding more quickly after transfer to a new environment were more likely to become dominant when paired with a conspecific, exhibited a lower cortisol response to a confinement stressor, and were more aggressive than trout that took longer to feed after transfer to a new environment (Overli et al., 2004). These results associated time to resume feeding post-stressor with indices of stress-coping style. A similar relationship appears to exist in zebrafish, where time to resume feeding after exposure to a standardized net stressor was significantly and

positively related to the cortisol response to the same stressor. Although the relationship was significant, it explained only 24% of the variation in time to resume feeding post-stressor, suggesting that factors other than stress-coping style influence the drive to feed in zebrafish. One such factor could be environmental temperature. Whereas salmonids inhabit temperate or cool waters (~12^{o}C), zebrafish are found at tropical temperatures (~28^{o}C). The higher temperatures experienced by zebrafish result in higher RMR and hence will require that more food be ingested to fuel resting metabolism. This argument suggests that the motivation to feed may be higher in zebrafish than in salmonids (Clarke and Johnston, 1999). The substantially faster resumption of feeding post-stress in the zebrafish of the present study that experienced somewhat restricted food availability supports this notion and suggests that time to resume feeding post-stress may be a less effective measure in assessing stress-coping style in zebrafish than in trout.

Regardless of whether the cortisol response to the stressor or the time to resume feeding post-stress was used as the indicator of stress-coping style, no significant relationship between stress-coping style and RMR was found in zebrafish. This result is in contrast to those obtained by Huntingford et al. (2010) where carp (*Cyprinus carpio*) classified as risk-taking were found to have significantly higher metabolic rates than risk-averse conspecifics. Risk-taking carp also exhibited greater competitive ability than risk-avoiding carp, and a dampened stress response (based on plasma glucose concentrations and corticosteroid receptor expression in response to a handling/saline injection stressor), placing risk-taking carp on the proactive end and risk-avoiding carp on the reactive end of the stress-coping style spectrum (Huntingford et al., 2010). Similarly risk-taking behaviour was positively correlated with RMR in juvenile European sea bass (*Dicentrarchus labrax*), although only following food deprivation (see below) (Killen et al., 2011). Huntingford et al. (2012) suggested that the linkages among risk-taking, bold, aggressive,

proactive behaviour (or risk-avoiding, shy, passive, reactive behaviour) can be explained as consequences of life history trade-offs between growth and mortality for life-styles that involve growing fast and maturing early versus those that involve slower growth and later maturation. Each strategy has costs and benefits that are context dependent, and the adoption of each strategy will bring with it a set of morphological and physiological specializations. For example, individuals aiming for higher growth rates will have higher metabolic rates and are more likely to possess large hearts and respiratory structures to help sustain the higher metabolic rate (Biro and Stamps, 2010). High growth rates must be fuelled by equally high rates of food acquisition, which means that individuals will need to be risk-takers (e.g. forage even in the presence of danger) and aggressive (to defend sites of food and resources). A positive association between RMR and risk-taking, bold, aggressive, proactive behaviour would be expected with this framework, as was observed in carp (Huntingford et al., 2010) and sea bass (Killen et al., 2011). By contrast, neither stress-coping style nor aggressive behaviour explained intra-individual variation in RMR in zebrafish in the present study.

A variety of explanations could account for the apparent absence of relationships between RMR and stress-coping style/aggression in zebrafish. One possibility is simply that these relationships, while present in other species, are not present in zebrafish. More data on a wider range of fish species are needed to address this possibility. However, recent work suggested the existence of relationships between bold-shy behaviour and stress-coping style in zebrafish (Oswald et al., 2012), as well as aggression and boldness (Moretz et al., 2007a), in keeping with the framework outlined by Huntingford et al. (2012). A second possibility is that relationships exist in zebrafish but were not detectable in the present study owing to insufficient resolution (the need for larger data sets to detect subtle differences in behaviour or RMR), the choice of

behavioural traits and/or indices of these traits that were used, or the experimental conditions. With respect to behavioural traits, focus on bold-shy (e.g. Moretz et al., 2007a; Dahlbom et al., 2011; Oswald et al., 2012) or risk-taking (Dugatkin et al., 2005) behaviours might prove more fruitful than the focus on aggression and stress-coping style adopted in the present study. The specific behaviours identified as measures of boldness also can influence the ability to detect relationships among behavioural traits (Moretz et al., 2007a) and, therefore, presumably between behavioural traits and RMR. With respect to experimental conditions, some evidence suggests that a restricted food regime can unmask relationships that were not apparent under conditions of abundant food supply. For example, the positive relationship between RMR and risk-taking behaviour in sea bass was apparent only after a period of food deprivation (Killen et al., 2011). Similarly, a significant relationship between RMR and time to resume feeding post-stress was apparent in the small group of zebrafish of the present study that were (inadvertently) subjected to a period of somewhat restricted feeding; individuals of higher mass-specific RMR resumed feeding significantly more quickly after the stressor than fish of lower RMR. Under conditions where food is restricted, individuals with higher metabolic rates may experience greater feeding motivation than individuals with lower metabolic rates. Numerous studies have documented differences in behaviour in food-deprived animals (e.g. Laland and Reader, 1999; Fraker, 2008; McCormick and Larson, 2008; Oliveira et al., 2011). For example, food deprivation in guppies (*Poecilia reticulata*) forced individuals to explore more and exploit problem-solving skills to locate a novel food source (Laland and Reader, 1999). In juvenile lingcod (*Ophidon elongatus*), increasing hunger levels resulted in individuals to emerge from shelters more readily, exposing them to higher risk than fish that were satiated (Oliveira et al., 2011). The observation that RMR was related to time to resume feeding post-stress in food-restricted zebrafish but not in zebrafish

provided with abundant food suggests that while aspects of behaviour or personality may contribute to individual variation in RMR, the extent of contribution is variable and context dependent. The effects of behaviour on RMR may not be detectable in well-fed animals, but variability in risk-taking/bold/aggressive behaviour driven by heightened feeding motivation may become apparent under conditions of food deprivation.

4.5 Relationships between organ mass and RMR

Whole-body RMR should reflect the sum of the mass-specific metabolic rates of all tissues multiplied by their masses. Organs such as the liver, heart, brain, intestine, kidney and gill (in fish) that are metabolically more active may contribute disproportionately to the RMR of the organism. Within endotherms, some studies have provided empirical support for this hypothesis (reviewed by Konarzewski and Ksiazek, 2013). For example, the masses of the intestine, kidney, liver and heart accounted for 52% of the variation in basal metabolic rate among six inbred strains of laboratory mice (Konarzewski and Diamond, 1995). In the present study, however, no significant relationship was detected between RMR and the masses of metabolically active organs such as the gill, heart or intestine plus liver. Similar findings were reported by Odell et al. (2003) for Trinidadian guppies (*Poecilia reticulata*), where little evidence was found to link maximum metabolic rate to the masses of heart or gill, and by Norin and Malte (2012) where RMR in brown trout was not related to the masses of the liver, heart, spleen, intestine or stomach. Thus, among ectotherms, the masses of metabolically-active organs do not appear to explain individual variation in RMR. Norin and Malte (2012) pointed out that visceral organ mass is a substantially smaller percentage of total body mass in fish than in endotherms. In fish, muscle, despite its low mass-specific resting metabolic rate, may contribute more to whole-

animal RMR than the visceral organs because it constitutes a major proportion of the individual's body mass (Norin and Malte, 2012).

Alternatively or additionally, differences in organ mass may be less important than differences in the mass-specific metabolic rates of the organs, i.e. differences at a molecular or enzymatic level may contribute to intra-specific variation in RMR. For example, Norin and Malte (2012) reported a significant relationship between RMR and the activity of two liver mitochondrial enzymes (cytochrome c oxidase and citrate synthase) in brown trout. Similarly, the activities of lactate dehydrogenase and malate dehydrogenase in skeletal muscle correlated positively to routine $\dot{M}O_2$ in marine teleosts (Childress and Somero, 1979). Intra-specific variation in metabolic rate also could be caused by differences in mitochondrial density among individuals, resulting in differences in the concentrations of enzymes responsible for aerobic metabolism. The degree of leakiness of the mitochondrial inner membrane to protons also may account for inter-individual variation in RMR (reviewed by Konarzewski and Ksiazek, 2013). Thus, RMR may be influenced by an array of factors beyond the size of metabolically-active organs and future studies should consider examining the influence of liver and white muscle enzyme activities on RMR.

4.6 Future directions

None of the factors investigated in the present study were found to reliably explain intra-specific variation of RMR in zebrafish. This conclusion emphasizes the need to investigate other potential contributors to variation in metabolic rate, but at the same time it is also possible that subtle relationships were not detectable in the present study owing to relatively low sample sizes for some experiments. For example, 39 zebrafish were used in the present study to investigate

whether a relationship existed between RMR and organ size, whereas Norin and Malte (2012) utilized 66 brown trout to the same end. In contrast to behavioural analyses performed in other studies (e.g. Careau et al., 2011; Killen et al., 2011), a relatively small pool of zebrafish (n = 20) was used to analyze the relationship between RMR and stress-coping style. Increasing the sample size in a number of experimental series in the present study, especially those involving behavioural analyses, might reveal relationships that were undetectable at lower sample sizes.

Future studies could also focus on additional factors that have the potential to explain individual variation in RMR. One such possibility is the environment in which early development occurs. Stressful conditions experienced by the mother can result in the production of eggs that contain high concentrations of cortisol, which in turn may elevate the RMR of offspring. For example, in a study by Giesing et al. (2011) on female three-spined stickleback (*Gasterosteus aculeatus*), individuals exposed to the threat of predation produced eggs that were larger and contained higher concentrations of cortisol than those of a control group. Relative to the control group, these eggs demonstrated a higher rate of oxygen consumption and, as juveniles, the offspring displayed tighter shoaling behaviour (anti-predator defense). The environment into which the eggs are laid also may have an impact on RMR. In a study on clownfish (*Amphiprion melanopus*), eggs laid on the periphery of the clutch demonstrated, on average, 24% lower RMR than those laid in the centre of the clutch (Green et al., 2006). The authors speculated that gradients in dissolved oxygen content might have influenced RMR as the individuals developed (Green et al., 2006). Parallel studies could be conducted in zebrafish to determine whether exposure of a female fish to a stressor influenced maternal hormone (e.g. cortisol) contributions to the developing eggs and/or hormone concentrations in offspring, and whether in turn these factors affect RMR when the offspring matured into adults. Factors such as

location in the clutch, water oxygen content during development, or the social environment in early development (e.g. Sloman and Baron, 2010) could also be investigated as influences on the RMR of individuals later in life. Other factors that could influence an individual's RMR during early growth and on into adulthood include challenges to the immune system and poor quality nutrition (Desai and Hales, 1997; Careau et al., 2010).

As noted above, the potential for hormones other than cortisol to influence RMR in zebrafish warrants investigation. Given the results of Ros et al. (2004) on the effects of 11-ketotestosterone on RMR in Mozambique tilapia, reproductive hormones would be an obvious first step. Although the present study found no significant link between RMR and the behavioural traits of aggression, activity and stress-coping strategies in zebafish, other behavioural syndromes or personality traits could be investigated. Huntingford et al. (2010) reported that common carp demonstrating risk-taking behaviour had higher metabolic rates than risk-avoiding carp. Hence, investigation of an individual's tendency to explore or take risks in a novel and potentially dangerous environment could be worthwhile.

4.7 Conclusions

It is evident from the results of the present study that intra-specific variation in RMR exists in zebrafish. However, the forces that drive this variation in zebrafish have yet to be determined. None of the factors tested in the present study (baseline cortisol levels, stress-coping style, organ mass, aggression, activity) could be identified as being significant contributors to variation in RMR. Hence, the results of the present study suggest that the RMR of an individual is a complex physiological trait that could be tied conditions in the environment during early development, hormonal status, past and/or present food availability, and personality traits.

Attempting to unravel these complex relationships to quantify the sources of variation among individuals in RMR is proving to be a difficult task

APPENDIX

Introduction

Social hierarchies have been observed to form in a variety of fish species in both natural and experimental settings (e.g. Kalleberg, 1958; Noakes and Letherland, 1977; Bachman, 1984; Nakano, 1995; Adams et al., 1998; Clement et al., 2005; Guderley and Couture, 2005; Colleter and Brown, 2011; Dahlbom et al., 2011). The formation of social hierarchies is based on an individual's ability to outcompete others in a group resulting in an unequal distribution of resources, in which dominant individuals claim a greater share (Fausch, 1984; Abbott and Dill, 1985; Huntingford et al., 1990; Gilmour et al., 2005). Such access to resources usually confers advantages to dominant individuals, which then actively and aggressively defend these resources from subordinate conspecifics. In contrast, subordinate individuals are negatively impacted by the formation of social hierarchies, demonstrating traits such as suppressed feeding and behavioural inhibition (reviewed in Gilmour et al., 2005).

Several studies performed on salmonid fish species suggested that metabolic rate is a reliable predictor of social status, where fish with standard metabolic rates (SMR) greater than expected on the basis of their mass became dominant over fish with SMR lower than expected on the basis of body mass (Metcalfe et al., 1995; Yamamoto et al., 1998; Cutts et al., 1999; McCarthy, 2001; reviewed by Gilmour et al., 2005). The mechanisms underlying this relationship remain unclear. Innate aggressiveness affects competitive ability (e.g. Holtby et al., 1993; Adams and Huntingford, 1996), and a link between aggression and SMR was established by Cutts et al., (1998). Using Atlantic salmon, Cutts et al., (1998) reported that as the proportion of "high" rSMR (rSMR or residual SMR is calculated from the difference between the observed and expected SMR of an individual given its mass) fish in a group increased, mean aggression

level also increased. Fish of higher SMR also may experience greater motivation to feed, driven by the greater energy input required to sustain high SMR (Johnsson et al., 1996).

However, there are a number of factors beyond SMR that may affect an individual's ability to attain dominant social status. For example, disparities in physiological factors such as energy reserves and condition factor also were found to affect the outcome of dyadic encounters (reviewed in Gilmour et al., 2005). In several salmonids, including rainbow trout (Abbott et al., 1985) and coho salmon (*Oncorhynchus kisutch*) (Rhodes and Quinn, 1998), differences in weight and/or length conferred dominant status to the larger individual. Personality traits, including innate aggressiveness and boldness, appear to influence social status. For example, in cooperatively breeding cichlids (*Neolamprologus pulcher*), individuals that demonstrated higher intrinsic aggressiveness (aggression was assessed by counting all agonistic behaviours displayed to a mirror for 5 min) were more likely to attain dominant status than their less aggressive conspecifics (Riebli et al., 2011). As noted above, innate aggressiveness in salmonids has been linked to competitive ability, which in turn may affect the ability to attain dominance (e.g. Holtby et al., 1993; Adams and Huntingford, 1996). Dahlbom et al. (2011) reported that in zebrafish, boldness was correlated with social status attained by an individual in a paired interaction. The outcome of social interactions also may depend on behavioural syndromes such as stress-coping strategy. In many animals, including salmonid fish, two distinct stress-coping styles exist (Koolhaas et al., 1999; Korte et al., 2005; Koolhaas et al., 2007). Pro-active individuals demonstrate a "fight-flight" response to a stressor that includes aggressive, bold and active behaviour as well as a relatively low cortisol response. The "freeze-hide" or reactive response encompasses shy, passive behaviour and a higher cortisol response to a stressor (reviewed in Koolhaas et al., 1999). In studies on rainbow trout lines selected for their response

to a standardized stressor to create "high" responders (HR) that exhibited post-stress cortisol levels that were significantly higher than those of "low" responders (LR), LR fish were successful in achieving dominance when paired with HR fish (Pottinger and Carrick, 2001). The mechanisms through which stress-coping style and success in social interactions are linked remain to be determined.

The initial goal of this project was to determine whether metabolic rate served as a reliable predictor of social status in zebrafish. If a relationship was found, the underlying mechanisms (such as feeding motivation, aggressiveness, and/or metabolic machinery) linking metabolic rate to social status were to be investigated.

Materials and Methods

Zebrafish (*Danio rerio*) were obtained from commercial suppliers (Big Al's, Ottawa, ON, Canada) and transported to the University of Ottawa Aquatic Facility where they were maintained in fibreglass tanks of either 3L or 10L volume. To reduce variation caused by potential differences between male and female fish, only female fish were used. Upon arrival at the Aquatic Facility, female fish were separated from male conspecifics. All tanks were maintained in a climate-controlled room ($28 \pm 1.5^{o}C$) and were supplied with flowing, dechloraminated city of Ottawa tap water maintained at $28 \pm 0.5^{o}C$. The photoperiod was held constant at 14L:10D. Zebrafish were fed three times a day with a mix of 50% Adult Zebrafish Complete Diet (Zeigler Bros Inc., Gardeners, PA, USA), 25% Golden Pearls Shrimp Larval Diet (Artemia International, LLC, Camille, AZ, USA), and 25% Spirulina Aquarium flake food (Burlingame, CA, USA).

Resting metabolic rate (RMR) of zebrafish was measured using intermittent closed-system respirometry (see Section 2.2 - Experimental Proctocols). Individuals were acclimated to the respirometry chambers overnight, with RMR measurements beginning the next day at noon. Three trials, each approximately two hours apart, were conducted from which the mean RMR was calculated. RMR measurements were carried out independently on two groups of 40 fish.

Following measurement for RMR of all fish in a group, pairs of fish were established in which a fish of "high" RMR (i.e. an individual that fell above the regression line of RMR vs. mass) was paired with a fish of "low" RMR of comparable mass from a different tank of fish (so that the fish were naive to each other). Each pair was placed in a behaviour arena, with the individual fish separated from one another by a perforated barrier for a 24 h recovery and acclimation period. The barrier was then removed, allowing the fish to interact, and behaviour was observed for 10 min three times per day (each observation separated by 3 hours) for 48 h, after which the fish in a pair were euthanized by anaesthetic overdose (ethyl *p*-aminobenzoate; 1g mL^{-1}). Social status was assigned on the basis of behaviour scores (for scoring system see Table A-1) and the fish within a pair with the higher behaviour score was deemed to be the dominant fish. During the last period of observation on the first day, each pair was fed a pinch of food, after which subsequent behaviours and interactions were recorded.

Table A.1. Scores assigned for specific behaviours observed within pairs of interacting female zebrafish (*Danio rerio*).

Behaviour	# of times observed	Score
Aggression (*Chases*)	0	0
	1-5	1
	6-10	2
	11-15	3
	15-20	4
	>20	5
Retreats	>10	0
	6-10	1
	1-5	2
	0	3
Activity	Immobile	0
	Restricted/spontaneous movement	5
	Constant patrolling	10

Results

Zebrafish used in these experiments ranged in mass from 0.45 to 0.90 g, averaging 0.65 ± 0.02 g (n = 32), and in length from 3.5 to 4.5 cm, averaging 3.9 ± 0.04 cm (n = 32). Absolute (untransformed) values of whole-animal RMR for these zebrafish ranged from 4.68 to 13.7 μmol h^{-1}, averaging 9.51 ± 0.37 μmol h^{-1} (n = 32).

From these fish, 16 pairs were generated. On average, the fish within a pair differed in RMR by 4.65 ± 0.18 μmol g^{-1} h^{-1} (n = 16) and in mass by 20 ± 3.3 mg (n = 16) and in length by 0.15 ± 0.02 cm (n = 16). Observations of behaviour over the 48 h interaction period were used to generate scores on the basis of which the fish within the pair were identified as dominant (average aggression score = 3.8 ± 0.2, average retreat score = 2.6 ± 0.1, n = 16) or subordinate (average aggression score = 0.5 ± 0.1, average retreat score = 0.4 ± 0.1, n = 16). In 9 of 16 pairs, the "high" RMR fish became dominant, a ratio that was not significantly different from that expected by chance (Chi-square analysis, $p > 0.05$). The aggression score each individual attained was plotted against its RMR, but no significant relationship was detected (Fig. A-1; Pearson Product moment correlation coefficient, $r = 0.170$, $p = 0.352$).

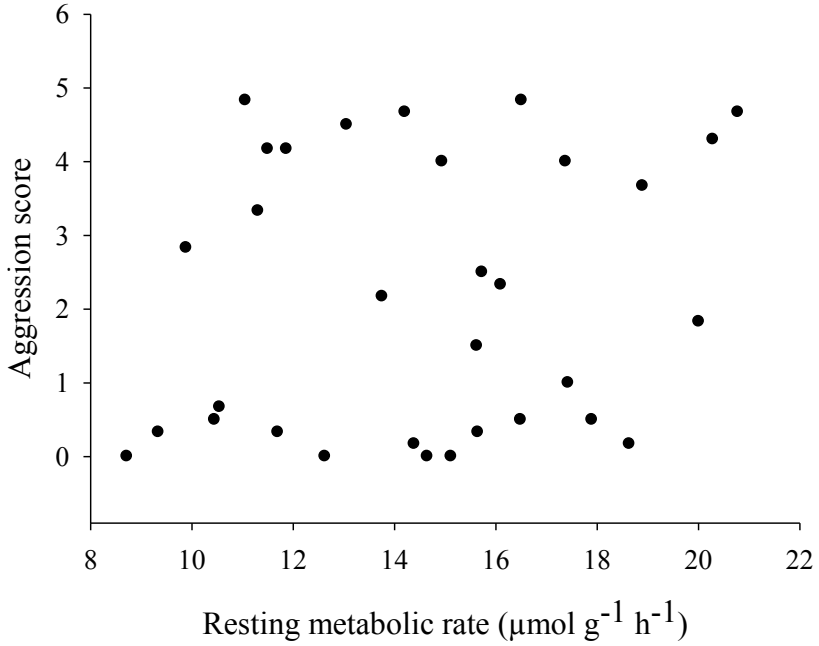


Figure A-1. Scores for aggression demonstrated by individual zebrafish (*Danio rerio*) during dyadic encounters are plotted against resting metabolic rate (RMR; measured as O_2 consumption by intermittent closed-system respirometry). No significant correlation was detected (Pearson product-moment correlation coefficient, $r = 0.170$, $p = 0.352$).

Discussion

As in previous studies on small groups of zebrafish (e.g. Larson et al., 2006; Filby et al., 2010; Dahlbom et al., 2011; Oliveira et al., 2011), pairs of zebrafish formed social hierarchies in which dominant fish could be distinguished from subordinate fish on the basis of their behaviour. Filby et al. (2010) reported that dominant and subordinate zebrafish differed not only in behaviour, but also on physiological and molecular levels. For example, significant differences in somatic growth were detected between dominant and subordinate zebrafish males, and were in turn attributed to suppression in subordinate males of hepatic expression of *igf1* (insulin-like growth factor), the main stimulator of somatic growth (Filby et al., 2010). Interestingly, *igf1* has been linked not only to somatic growth (Vera Cruz et al., 2006), but also to aggression and dominant behaviour displayed by Nile tilapia (*Oreochromis niloticus*) (Vera Cruz and Brown, 2007). Because the establishment of a social hierarchy requires aggressive acts, all individuals are expected to experience stress (i.e. high circulating cortisol concentrations) during hierarchy formation. However, in dominant individuals, cortisol concentrations returned to unstressed levels within a few hours of hierarchy formation, whereas circulating cortisol concentrations remained elevated in subordinate individuals for longer periods of time (Overli et al., 1999; Sloman et al., 2000), a situation that is indicative of chronic stress (Sloman et al., 2002). Although these studies focused on salmonid species, a similar situation appears to exist in zebrafish in that subordinate zebrafish exhibited significantly higher cortisol concentrations than dominant fish (Filby et al., 2010). The elevated cortisol concentrations in subordinate males were associated with elevated expression of *crh* (corticotrophin-releasing hormone), *npy* (neuropeptide y), and *gr* (glucocorticoid receptor). Corticotropic releasing factor and NPY are well known stimulators of adrenocorticotropic hormone (ACTH), the primary stimulator of cortisol synthesis

and secretion, and their transcripts have been found to be up-regulated by many stressors (Wahlestedt et al., 1987; Mommsen et al., 1999), including social stress (Doyon et al., 2003). Given the impact that social interactions may have on the health and fitness of an individual fish, it is useful to investigate potential predictors of social status in zebrafish.

Larger body size in zebrafish was found to be a good predictor of an individual's ability to win fights and hence gain dominant social status (Hamilton and Dill, 2002; Pyron, 2003; Spence and Smith, 2005; Filby et al., 2010), but between fish that are evenly matched in size, predicting which fish will gain dominant status is more difficult. In several salmonid species, RMR that was higher than average on the basis of mass was found to be a reliable predictor of dominant status in dyadic interactions with fish of average or lower than average RMR (Metcalfe et al., 1995; Yamamoto et al., 1998; Cutts et al., 1999; McCarthy, 2001; reviewed by Gilmour et al., 2005). The results of the present study, however, indicated that RMR was not an effective predictor of social status in zebrafish. Zebrafish of "high" RMR were no more successful than expected by chance in securing dominant social status when paired with an individual of "low" RMR. Whether the predictive power of RMR for social status is restricted to juvenile salmonids or occurs more broadly across fish species that form social hierarchies (but not in zebrafish) remains to be determined. Consideration of differences between zebrafish and salmonids may shed light on this question.

In their natural environment, juvenile salmonids compete to establish feeding territories (Li and Brocksen, 1977; Sloman et al., 2000; Sloman et al., 2001). The outcome of competition for this limited resource can incur severe negative consequences for subordinate individuals, including reduced growth rate and often increased mortality, in both natural and lab settings (Abbott and Dill, 1985; Metcalfe et al., 1990; McCarthy et al., 1992; Pottinger and Pickering,

1992; Sloman et al., 2000; Sloman et al., 2001). For example, the food intake of subordinate rainbow trout did not match that of dominant fish, even when the fish were separated for feeding so that the subordinate fish had access to food (DiBattista et al., 2006). Moreover, Arctic charr continued to display a reduction in food intake even after being separated from their dominant conspecific (Jobling and Wandsvik, 1983). In both cases, reduced feeding of subordinate fish was interpreted to be stress-induced anorexia (Winberg and Nilsson, 1993; Overli et al., 1998; Overli et al., 1999). Unlike the behaviour that has been observed in salmonids, subordinate zebrafish were not prevented from feeding by aggressive defence of the food source by dominant individuals. Such inter-species variation in behaviour may reflect differences in the life stages at which the two species are typically studied, namely for zebrafish, reproductively mature adults which exhibit strong schooling behaviour and for rainbow trout, juveniles that exhibit aggressive, territorial behaviour (Dahlbom et al., 2011). Moreover, social hierarchies established among sexually mature adults may, in addition to competition for food and shelter, reflect competition for preferential access to mates (Francis et al., 1993).

Zebrafish also differ from salmonids in inhabiting warmer water temperatures (28°C) and consequently demonstrate higher mass-specific metabolic rates than salmonids, which inhabit temperate/cool waters (12°C). In order to sustain a higher resting metabolic rate, zebrafish also require more food per unit time (per unit mass) than salmonids (reviewed by Clarke and Johnston, 1999). The impact of factors such as RMR and motivation to feed on competitive ability may be dampened in an experimental setting in which abundant food is supplied, such as the present study, if the relationship between SMR and dominance is context dependent. For example, in an environment where food availability is limited, individuals of lower RMR are more likely to do well because of their lower cost of maintenance (Metcalfe, 1986). Acquisition

of dominant status also may be affected by food availability; for example, low food availability may trigger increased aggression during competition for food (e.g. Andersson and Ahlund, 1991; Lemel and Wallin, 1993).

In zebrafish, factors other than RMR appear to be the main determinant of social status. Moretz et al. (2007b) reported that social behaviour in zebrafish may be heavily influenced by strain differences and the environment in which early development occurred. To test this hypothesis, Moretz et al. (2007b) used three different strains of zebrafish. The first, TM1, was a strain derived from fish that were acquired from a pet store in 1986 and were 30 generations removed from the point of the experiment (Robison and Rowland, 2005). Individuals from the second strain, Nadia, were only 5 generations removed from wild-caught fish. These fish displayed several morphological and physiological differences from the TM1 strain that suggested that they had not yet evolved in response to the laboratory environment (Robison and Rowland, 2005). The final strain, SH or Scientific Hatcheries, was similar to TM1 and was commercially available from Scientific Hatcheries, Huntington Beach, CA. However, in contrast to the TM1 strain that was maintained by breeding small numbers of individuals, SH fish were reared and bred in large numbers (similar to trout hatcheries). Hence, each of these strains evolved under different selective regimes (Moretz et al., 2007b), and the fish also exhibited differences in behaviour. Individuals from the TM1 strain were more social, more likely to approach a predator and also took less time to recover from a disturbance than the other two strains. In addition, individuals from the TM1 and Nadia strains raised in mixed-strain groups were more aggressive (attempted to bite a mirror image more often) than those raised in pure-strain groups (Moretz et al., 2007b), suggesting that individuals brought up in a mixed-strain environment would be at an advantage in attaining dominance over fish raised in a pure-strain

environment. However, this result was independent of the stage of mixing (i.e. whether they were mixed as juveniles or adults), which suggested that aggression is a malleable trait. Aggression has been found to change based on factors such as residency status (Leimar and Enquist, 1984), relative size (Moretz, 2003), breeding condition (Guiasu and Dunham, 1997), and motivational state (Cremer and Greenfield, 1998). The fish used in the present study were purchased as adults from a fish supplier, so the rearing environment, as well as strain, was unknown.

Personality traits such as boldness also may influence the outcome of social interactions. For example, Dahlbom et al. (2011) reported that boldness was significantly correlated to the social status zebrafish attained in a dyadic interaction. Boldness was assessed by monitoring an individual's behaviour in three different contexts; a novel environment with no roof, the same environment with a roof, and an environment with no roof but an unfamiliar object in its place. The boldness of fish in these environments was quantified by measuring activity (distance moved), time spent out of shelter, and thigmotaxis (staying close to the walls of the arena). Subordinates were described as being initially bold in regards to swimming distance and time spent next to a novel object. However, individuals that eventually became dominant demonstrated bold behaviour only in the later phases of the experiment (Dahlbom et al., 2011). These observations led to the hypothesis that shy animals increased activity initially in an attempt to find an escape route. Bolder animals, on the other hand, may have assessed the situation while in an immobile state, beginning careful exploration only after the situation was interpreted as being safe. Dahlbom et al. (2011) also discovered that, as a measure of boldness, the time an individual spent in the middle of the tank during the open field test was correlated to the social status attained in a dyadic interaction.

Finally, social status attained in dyadic interactions may be affected by stress-coping style. In a study on juvenile rainbow trout (Overli et al., 2004), fish that attained social dominance were observed to be the first to resume feeding after transfer to isolation (a handling stressor). The results suggested that appetite inhibition after transfer to a new environment, a reflection of the physiological response to stress, might also be useful in predicting the outcome of social interactions. Rainbow trout that demonstrated lower cortisol responses to a 30 min confinement stressor were found to display more aggressive behaviour when paired with a conspecific (Overli et al., 2004). It would be worthwhile to determine whether a relationship exists between stress-coping style and social status in zebrafish.

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