Evaluating the use of telomere length for the assessment of cumulative stress in zebrafish (*Danio rerio*)

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Abstract

Zebrafish (*Danio rerio*) are used in their millions worldwide for scientific research and testing. Despite their popularity as an animal model, their welfare has not been well-considered until recently. In particular, the role of environmental enrichment for improving zebrafish welfare is increasingly being examined. However, the welfare assessment indices available to researchers are limited, particularly for studies of long-term conditions such as housing. Telomeres are protective caps on the end of chromosomes that shorten during cell division and thus provide an indication of biological ageing. Recent research has demonstrated that telomeres shorten faster during stress. Thus, telomere length may be a useful marker of cumulative and chronic stress and thus serve as an indicator of an animal's longer-term state of welfare. The aim of this study was to assess the response of telomeres to cumulative stress in zebrafish, to establish whether this marker could be used for future welfare assessment in this species. 57 fish were exposed to an Unpredictable Chronic Stress protocol for four weeks, while 57 non-stressed controls were maintained under identical, industry standard conditions. After this time, the telomere length of a mixed-tissue sample taken from fish in each group was compared. Whole-body cortisol concentration was also measured to evaluate whether any change in telomere length was correlated with a physiological stress response. I hypothesized that telomere length would be shorter and cortisol concentration higher in the stressed group. Contrary to expectations, there was no difference in telomere length between stressed and control fish. Nor was there a difference in cortisol concentration, suggesting that either the fish were not sufficiently stressed, or that a ceiling effect had been reached. The most likely reason for this is that the stress treatment selected was not stressful enough to induce a measurable response. However, an alternative explanation is that the rate of telomere shortening was masked by the activity of telomerase, an enzyme that maintains telomere length in this species. Future explorations of the effect of stress in telomere dynamics should include evaluation of both length and telomerase activity. An effect of fish sex on telomere length was found, with females having shorter telomeres than males. Although these results cannot be used to confirm the utility of telomere length as a welfare indicator, they raise an interesting and thus far unexplored question of the role that sex plays in telomere maintenance.

This thesis is dedicated to my Nana Lyn.
Thank you for always fostering my curiosity, encouraging my thinking, and supporting my passion.
Love always, Morgan.

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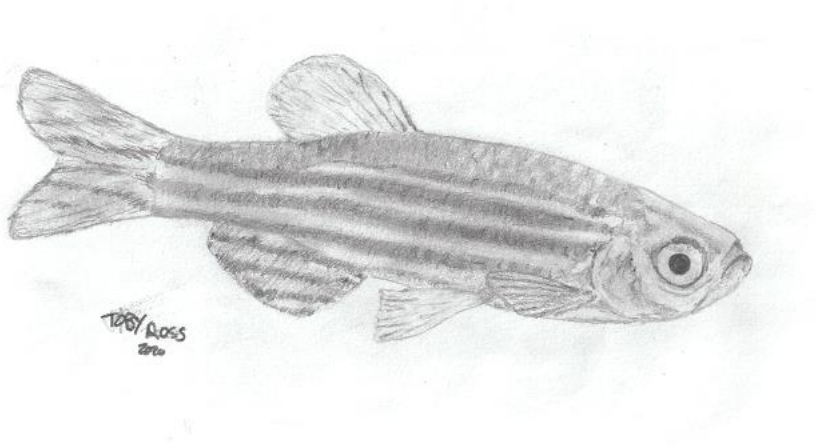

In acknowledgement of the animals used in this research.

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1 Introduction

Fish are one of the most popular animal models for research in the world, second only to mice in some countries (Canadian Council on Animal Care 2018; Home Office 2018). Zebrafish (*Danio rerio*) were first used for research in the 1970s and since then have become the prominent fish species used for biomedical and toxicology research. Due to their small size, fast growth rate, short generation time, and transparent egg that allow high-throughput testing and real-time monitoring of embryological development, an estimated 5 million zebrafish are used for research every year (Lidster *et al.* 2017). Despite this, zebrafish husbandry and welfare have only recently become areas of scientific interest.

Animals used for scientific research and testing should be kept in a way that optimises their welfare. This is important not only for ethical reasons, but also for the maintenance of 'social licence' to continue using animals for research. However, due to the need to keep large numbers of fish economically, zebrafish are kept in large groups in small tanks. In addition, zebrafish tanks are often kept completely barren, generally for greater economy of care (Williams *et al.* 2009). Environmental barrenness may cause welfare compromise in zebrafish, as it does in other laboratory species (Graham *et al.* 2018a). In addition, barrenness may also influence or confound experimental results. Thus, there is a need to investigate the welfare state of zebrafish kept under standard laboratory conditions.

Currently, most studies investigating fish welfare focus on "stress", and evidence of stress is considered to be evidence of welfare compromise. The most commonly used

stress indicators for fish are either behavioural or those that reflect an aspect of the primary physiological stress response (e.g. cortisol concentration). However, these indicators are often too general (in the case of cortisol) or too context-dependent (behaviour) to support interpretation of welfare state or allow comparison among treatments or situations. In addition, such measures are acute indicators, meaning that they provide information about the immediate (behaviour) or recent (cortisol) state of the animal at the time of observation. However, for reasons that will be discussed, they are less useful for understanding the experience of the animal over an extended period of time. When assessing the effects of environment or husbandry practices on welfare, the indicator selected must represent the state of the animal over the longer-term. Thus, there is a need for validated welfare indicators that provide information about the longer-term experiences of an animal.

Recently, the rate of telomere attrition (shortening) has been suggested for this purpose (Bateson 2015). Telomeres are "caps" on the ends of chromosomes that shorten during cell division in a process called attrition. When telomeres reach a critically short length, they trigger cellular senescence pathways that result in the cell being unable to further divide. An increase in the proportion of senescent cells in a tissue is indicative of tissue ageing. Therefore, the rate of telomere attrition is considered an important biomarker of ageing. In addition, recent research across vertebrate species suggests that the rate of attrition is increased by stress. The aim of this study was to explore whether exposure to a chronic intermittent stress treatment would cause increased telomere shortening, in order to establish the usefulness of telomere attrition as an animal welfare indicator.

In the following sections, I will provide detailed background on the use of zebrafish in scientific research and how current maintenance conditions have the potential to cause welfare compromise in these animals. I will then discuss current markers used for assessing the welfare of fish, and why these markers are not sufficient for answering questions about zebrafish welfare in the long-term. Finally, I will discuss current knowledge of telomere dynamics and how they respond to stress in other species, to illustrate the potential value of telomere attrition for assessing longer-term welfare in zebrafish.

1.1. Use of zebrafish for scientific research and testing

Zebrafish are the model species of choice for a wide range of scientific disciplines, from biomedical research concerned with processes like ageing, cognition and stress, to toxicology testing (Steenbergen *et al.* 2011; Dai *et al.* 2014; Van Houcke *et al.* 2015; Meshalkina *et al.* 2017). The popularity of zebrafish can be attributed to many features. In particular, they are well suited to high-throughput research and are economic to house and maintain.

1.1.1. Zebrafish are ideal for high-throughput research

The reproductive and developmental biology of zebrafish make them ideal for high-throughput research. Zebrafish are oviparous and release eggs that can be collected non-invasively in large numbers. These eggs are highly amenable to genetic modification and are transparent, allowing researchers to visually monitor embryological development (Steenbergen *et al.* 2011). In addition, zebrafish eggs

develop and hatch within 30 hours, so embryological development can be directly observed in real-time.

Zebrafish also have a fast growth rate and reach sexual maturity quickly, allowing researchers to monitor development over the course of weeks. Zebrafish develop from newly hatched to adult in only 12 weeks, with a total lifespan of approximately 5 years (Parichy *et al.* 2009; Reed and Jennings 2011; Froehlich *et al.* 2013). Juveniles reach sexual maturity at approximately 12 weeks post-fertilisation, and from there females can produce hundreds of eggs every few days (Steenbergen *et al.* 2011). This feature is particularly useful for genetics researchers wishing to observe effects over multiple generations. Together, these features mean that large numbers of zebrafish can be produced cheaply over a short time-period. However, producing large numbers of animals requires housing large numbers of animals.

1.1.2. Zebrafish can be maintained economically

Another important consideration for selecting a research species is the cost of maintaining the animals and facilities. Zebrafish are a tropical freshwater species that require a water temperature around 28°C and strict control of other water parameters including pH, nitrate, and salinity, requiring multiple water filtration mediums (Reed and Jennings 2011). However, they are small, socially gregarious fish that display low aggression in groups (Suriyampola *et al.* 2015). This means they can be kept in small tanks with high stocking densities. Most industrial facilities keep zebrafish in tanks sized between 3.5 and 8 litres, at densities of approximately five to ten fish per litre (Matthews *et al.* 2002; Reed and Jennings 2011; Lawrence and Mason 2012). As an aquatic species, zebrafish do not require bedding, bowls, or other objects as part

of their day-to-day care. Thus, zebrafish can be kept in very large numbers relatively cheaply compared to other common laboratory animals such as rodents.

1.1.3. Barren tanks are used for practical and economic reasons

Zebrafish are usually kept in barren tanks, which provide a number of benefits in a research environment. One of the main priorities for the design of animal housing is ease of monitoring. Zebrafish are fast-moving animals with a propensity to hide behind in-tank objects. Consequently, the health of fish can be better monitored in a barren tank (Wilkes *et al.* 2012).

Another priority for tank design is ensuring that objects within tanks do not interfere with research outcomes. Because zebrafish are widely used for toxicology research and testing, it is important that all objects within tanks are chemically inert (Wilkes *et al.* 2012). That is, if objects are to be added to the tank, they must be made of a material that cannot interact with chemical elements that are introduced to the tank for research purposes. The fewer objects added to a tank, the less material there is to potentially react. This makes barrenness the best option for toxicology research. Similarly, in-tank objects increase the surface area available for algal and microbiological growth, so barren tanks are easier to keep clean.

Despite the practical and economic benefits of keeping zebrafish tanks bare, barren environments have the potential to compromise fish welfare (see 1.2.2 below). The welfare of animals used for scientific research and testing is important for the sake of the animals themselves (Fraser *et al.* 1997). Having good welfare is just as important to animals on their terms, as it is to humans on our terms (de Vere and Kuczaj 2016).

Thus, there is an ethical obligation for researchers to ensure that the animals they use have the best welfare possible. In addition, it is important to ensure good animal welfare in order to maintain 'social licence', or the general acceptance of society that allows animals to be used for research and testing (Mellor and Reid 1994).

1.2 Zebrafish welfare

1.2.1 A brief characterisation of animal welfare

Welfare is considered to be an animal's internal state, reflecting how it experiences its world. Welfare state at any point in time reflects the multiple mental experiences (affects, emotions, feelings) an animal is having (Webster 2016). Affective experiences are those that *mean* something to the animal. Specific affective experiences can be negative (welfare compromising) or positive (welfare enhancing) (Mellor and Beausoleil 2015). Negative affects may be generated due to a disturbance of physiological function within the body (e.g. tissue damage leading to pain, dehydration leading to thirst) or may be stimulated by the animal's perception of its external environment (e.g. fear, anxiety) (Mellor 2017). Positive affects such as feelings of safety, companionship and engagement result from the animal's perception of its environment and opportunities to engage in strongly motivated behaviours (Mellor 2015). Affective experiences are thought to confer a fitness advantage to the animal by providing long-lasting motivation to avoid (in the case of negative experiences) or seek out (in the case of positive ones) in the future the situation that generated the initial experience (Elwood 2011).

In order to have such mental experiences, an animal must have sufficient neural connectivity and function to produce and maintain a subjective internal state. That

is, the animal must be sentient and conscious (Mellor 2016). All vertebrates, including fish after the larval stage of development, are considered to be sentient in New Zealand law (Animal Welfare Act 1999). Therefore, fish are considered to be able to have mental experiences that they can interpret as good or bad, and as such their welfare must be considered (Sneddon *et al.* 2018).

1.2.2 Environmental barrenness may compromise zebrafish welfare

Although laboratory fish welfare has only recently become an area of inquiry, there is evidence that environmental barrenness causes welfare compromise in rats and mice (Bayne 2018). Evidence of welfare compromise in barren-housed rodents includes indications of behavioural restriction, and increased anxiety. For example, rodents perform strongly-motivated behaviours such as digging and burrowing when provided with appropriate substrate (Makowska and Weary 2016), and barren housing thwarts the ability express these behaviours. In addition, rats housed in an enriched environment display less evidence of anxiety than barren-housed rats (Harris *et al.* 2009).

In zebrafish, there is some evidence that barren housing influences behaviour, but what those behavioural changes mean in terms of welfare is unclear. For example, zebrafish show more locomotion and erratic movement in barren tanks than in tanks with added objects; such behaviour is generally assumed to be indicative of increased anxiety (Speedie and Gerlai 2008; von Krogh *et al.* 2010). In addition, the only stereotypic behaviour characterised in zebrafish (waving) has only been observed in barren tanks (Kistler *et al.* 2011). However, there is a lack of information on

physiological responses to aid in interpreting behavioural evidence, making it challenging to draw well validated conclusions about the impact of barren housing on zebrafish welfare.

One of the main challenges for assessing how features of the environment, such as barrenness, influence welfare is that animals are exposed to them for long periods of time. For example, zebrafish that are used for breeding (and therefore not used for research) will spend their entire lives in barren tanks. This means that welfare indicators that provide information about the immediate state of the animal (such as behaviour) will not provide enough information to infer the welfare state over periods of weeks to years.

1.2.3 Indicators of welfare states

Affective states are subjective to the animal and occur internally, preventing their direct observation or measurement. However, these states can be indirectly inferred through the use of scientifically validated indicators that can be observed or measured (Mellor 2017). To make this even more challenging, fish live in a very different world to humans, and this limits our understanding of fish affective experiences . For example, we cannot know what fish experience when they are exposed to an inappropriate water pH. However, we can infer through physical and behavioural indicators whether this experience is interpreted as good or bad by the animal itself (Mellor *et al.* 2009).

The affective experiences that are currently the best validated and most accepted in fish are those that arise from compromised health or physical state, such as pain

(Sneddon *et al.* 2018). These experiences can be clearly inferred through observation of physical, physiological, or behavioural indicators. Other affects, such as frustration, anger, and boredom, recognition of which is not yet possible, are not often discussed in the fish welfare literature. Because such affects cannot yet be identified in fish with any degree of confidence, they are not yet useful experiences to discuss when assessing fish welfare. It should be noted that a lack of discussion around these affects does not imply that they do not exist in fish. However, most currently accepted fish welfare indicators reflect affective states that can be more clearly inferred.

1.2.4 Types of welfare indicators

Welfare indicators can be related to the resources available to an animal, management practices applied, or the state of the animal itself. Resource-based indicators represent the risk that features of the environment pose to the welfare of animals thus kept, rather than representing welfare state directly (Beausoleil and Mellor 2017; Harvey *et al.* 2020). As such, resource-based indicators can sometimes be used to infer physical health or nutritional status but only if their effect on animals has been firmly demonstrated (Beausoleil and Mellor 2017).

Resource-based welfare indicators for fish include water quality (e.g. measures of dissolved oxygen, nitrate, or carbon dioxide), water temperature, and food availability (Braithwaite 2017). However, most of these measures are made at population-level and do not provide information about the welfare of individual fish. In a group of animals, it is unlikely that the welfare state of all individuals is the same. For example, an individual may have a physical injury, be restricted from access to

resources by its conspecifics, or be inherently fearful than other animals in the group. Therefore, it is important to be able to assess the welfare of individual animals.

The most direct indicators of welfare are animal-based because these indicators focus on the individual animal and its current state (Harvey *et al.* 2020). These indicators are also the most useful for assessing the welfare of individuals in group-living situations such as in farming or laboratory tanks.

For the reasons explained above, in aquaculture, there is a strong focus on the physical health and function aspects of welfare. Most fish welfare assessments focus on physical or physiological indicators, and fish are commonly considered to have acceptable welfare if they are physically healthy (Turnbull *et al.* 2005; Ellis *et al.* 2012; Toni *et al.* 2019). In particular, there is a strong emphasis on avoidance of "stress" to ensure acceptable welfare in fish. A fish that is stressed is considered to have poor welfare due to the downstream effects of chronic stress on productivity measures such as growth and reproduction (Braithwaite 2017).

Some animal-based welfare indicators used for mammals and birds are technically difficult or unfeasible for use on fish. Examples include electroencephalograms (to measure electrical brain activity), qualitative behavioural assessment, or eye temperature measurements. This may be due to equipment requiring a dry body surface or not being suitable for use underwater, differences in behaviour (e.g. fish do not have identifiable facial expressions), or inability to safely handle the animals. Thus, the development of feasible, valid welfare indicators for fish is a challenging task. Currently, the two main categories of fish welfare indicators in use for the

assessment of zebrafish welfare are physiological stress and quantitative behavioural assessments (Wilkes *et al.* 2012; Pavlidis *et al.* 2015; Graham *et al.* 2018a).

1.2.5 Fish welfare can be assessed by evaluating physiological stress

Animals live in dynamic environments that demand mechanisms to cope with changing conditions (Gorissen and Flik 2016). The group of mechanisms present in vertebrates that are responsible for this physiological adaptability are collectively termed the stress response (Wendelaar Bonga 1997). Deviations in the environment or internal physical state that lead to a disturbance of physiological homeostasis are called stressors. Stressors are perceived by a variety of sensory receptors and that information is integrated within the animal's brain. This leads to the initiation of an adaptive physiological response, with associated behavioural responses and, in some cases, generation of affective experiences and cognitive responses (Wofford and Goodwin 2002).

Thus, components of the stress response can be characterized as psychological or physiological. The psychological component involves the initiation of a cognitive response which leads to the performance of a behaviour aimed at avoiding or removing the stressor (such as running away). This also generates a related affective experience such as acute pain or fear (Wofford and Goodwin 2002; Dedovic *et al.* 2009). The physiological component involves the activation of the sympathetic nervous system and release of hormones that increase the amount of free energy available to support the behavioural response, as well as any other physiological changes necessary to promote a return to homeostasis (such as an immune response

in the case of physical injury) (Schreck and Tort 2016). Therefore, the physiological stress response can often be used to infer the generation of an affective state related to perception of the challenge (Moberg and Mench 2000). While the psychological components of stress are challenging to evaluate in non-human animals, physiological stress can be measured directly using a number of different factors in the response pathway.

The vertebrate physiological stress response occurs in three stages. The primary stage is the activation of the neuroendocrine axes that lead to the release of catecholamines and glucocorticoids. The secondary stage is the immediate or short-term physiological effect of these compounds. Finally, the tertiary stage is the long-term effect of these compounds on the animal as a whole (Schreck and Tort 2016). The three stages of the stress response are dynamic and interactive, and may not occur sequentially (Barton 2002).

1.2.5.1 Primary stress response

The primary stage begins with the recognition of a threat to homeostasis (Barton and Iwama 1991) which activates two neuroendocrine axes originating in the hypothalamus. In fish, these are the sympathetic-chromaffin (SC) axis, and the hypothalamic-pituitary-interrenal (HPI) axis (Gesto *et al.* 2015; Madaro *et al.* 2015). Their activation respectively results in the release of catecholamines from the chromaffin cells and cortisol from the interrenal cells of the head kidney (Barton and Iwama 1991; Arends *et al.* 1999; Nardocci *et al.* 2014).

The perception of a threat activates the SC axis by stimulating pre-ganglionic cholinergic fibres of sympathetic nerves originating in the hypothalamus (Wendelaar Bonga 1997; Reid *et al.* 1998; Bernier *et al.* 2009). These fibres mediate the release of dopamine, the precursor to adrenaline, as well as adrenaline itself and noradrenaline from the chromaffin cells (Barton and Iwama 1991; Schreck and Tort 2016). These cells are found predominately in the head kidney, specifically in the walls of the posterior cardinal vein (Barton and Iwama 1991; Wendelaar Bonga 1997; Nardocci *et al.* 2014). This is a fast and transient response which occurs within seconds to minutes of stressor perception, and it is difficult to measure baseline activity without stimulating it (Barton 2002; Ellis *et al.* 2012). Thus, SC axis activation is not often directly measured for the purpose of welfare assessment. For this reason, the remainder of this review focusses on the activation of the HPI axis.

When the HPI axis is activated, neurons originating in the hypothalamic nucleus preopticus send direct projections to the pituitary pars distalis and pars intermedia (Steenbergen *et al.* 2011; Nardocci *et al.* 2014; Winberg *et al.* 2016). When a stress response is initiated, these axons secrete corticotrophin-releasing factor (CRF) and arginine vasopressin (AVP) (Kulczykowska 2001; Almeida *et al.* 2012). CRF acts in the pars distalis to stimulate the conversion of pro-opiomelanocortin (POMC) protein into adrenocorticotrophic hormone (ACTH) (Madaro *et al.* 2015). In the pars intermedia, CRF acts in concert with AVP to convert POMC into α-melanocyte-stimulating hormone (α-MSH) and β-endorphin (Wendelaar Bonga 1997; Harris and Bird 1998; Gorissen and Flik 2016). ACTH, α-MSH and β-endorphin are subsequently released into the peripheral circulation, where they act upon the interrenal cells of the head kidney to stimulate the synthesis and release of cortisol,

the primary glucocorticoid of fish (Wendelaar Bonga 1997; Bernier *et al.* 2009; Steenbergen *et al.* 2011; Takahashi *et al.* 2013; Madaro *et al.* 2015; Sadoul and Geffroy 2019). This system is analogous to the hypothalamic-pituitary-adrenal axis in mammals (Steenbergen *et al.* 2011; Sadoul and Geffroy 2019).

1.2.5.2 Secondary stress response

Cortisol functions as a glucocorticoid to modulate energy metabolism by stimulating a range of catabolic responses. This increases the availability of free energy sources for immediate use (Barcellos *et al.* 2010). In addition, cortisol functions as a mineralocorticoid in teleost fish (Wendelaar Bonga 1997; Gilmour 2005; Bury and Sturm 2007). Glucocorticoid receptors and mineralocorticoid receptors are ligand-binding transcription factors which are both able to bind cortisol, allowing it to function appropriately in both energy and mineral homeostasis (Prunet *et al.* 2006; Bury and Sturm 2007; Stolte *et al.* 2008; Gorissen and Flik 2016). For example, increased circulating cortisol results in greater tolerance to changes in salinity in the short term (McCormick 2001; Metz *et al.* 2003; Prunet *et al.* 2006).

Cortisol also modulates the immune response. Although it is classically considered to be an immunosuppressive hormone, some of its functions could be interpreted as adaptive, particularly in acute stress (Yarahmadi *et al.* 2016). For example, after injury, the localised immune response to infection is enhanced by preferential trafficking of lymphocytes to the affected area (Yada and Tort 2016). In contrast, fish experiencing chronic overcrowding stress show a decrease in immune function including reduced total serum protein, lysozyme activity, phagocytic activity and cytokine gene expression (Yin *et al.* 1995; Dhabhar 2006; Yarahmadi *et al.* 2016).

Finally, cortisol plays a regulatory role in the production of reactive oxygen species (ROS) and antioxidant gene expression. Cellular oxidative stress occurs when the balance between ROS production and antioxidant defence mechanisms breaks down, leading to an excess of ROS (Haussmann and Marchetto 2010). Although cortisol is involved in promoting both ROS production and antioxidant protection, its overall effect is determined by the duration of the stress response. A meta-analysis of studies addressing relationships between glucocorticoids and oxidative stress in vertebrates, found that in the short-term (up to five days), cortisol stimulated antioxidant production, but after three weeks of exogenous glucocorticoid treatment oxidative stress increased strongly (Costantini *et al.* 2011). This trend of short-term adaptive advantages and long-term damage is consistent for many of cortisol's roles.

1.2.5.3 Tertiary stress response

The tertiary stage is the long-term and downstream effects of the secondary response on the animal as a whole (Schreck and Tort 2016). These consequences include inhibitory effects on reproduction, growth and immune function (Barton and Iwama 1991; Schreck and Tort 2016). The tertiary response is largely due to 'allostatic overload', that is, the ongoing energetic cost of the stress response. It occurs when the primary and secondary responses fail to re-establish homeostasis (Galhardo and Oliveira 2009; Schreck and Tort 2016). It is thought that the function of this inhibition is to divert energy away from non-essential processes to aid survival when the animal is under stress (Branson 2008).

1.2.5.4 Characterization of the stress response according to stressor duration, pattern and intensity

When designing or selecting an experimental stress protocol, it is important to understand how the stress response can be modulated in order to predict the likely effects of the chosen protocol on the welfare indicators measured. The overall biological effect of the stress response is dependent on four main factors related to the stressor: it's duration, intensity, predictability and controllability (Dhabhar 2006; Korte *et al.* 2007; Galhardo and Oliveira 2009; Winberg *et al.* 2016).

In general, stress can be classed as either acute or chronic depending on the duration of exposure to a stressor. Short-term (acute) stressor exposure, occurring over a timespan of seconds to hours, causes acute stress (Ellis *et al.* 2012). When acute stress occurs, homeostasis can be re-established after the termination of stressor exposure, so only the primary and secondary stages of the stress response are stimulated. In contrast, long-term stressor exposure can cause *chronic* or *cumulative* stress, depending on the manner of exposure (Schreck 2000).

When a long-term stressor is applied continuously, this causes *chronic* stress. During chronic stress, there is no period of rest or recovery, thus homeostasis cannot be restored before the stimulation of the tertiary stage of the stress response (Arjona *et al.* 2009). In other words, during chronic stress the effects of cortisol persist long enough to impair physiological functions such as growth and immunity. Chronic stress also results in a general desensitisation (or down-regulation) of the HPI axis, meaning that the animal is less able to mount a physiological response to additional acute stressors (Ellis *et al.* 2012). Importantly, when HPI down-regulation occurs, tertiary stress processes can persist, even in the absence of high circulating levels of

cortisol. This is due to changes in immune function mediated by cytokines (such as inflammation) and other downstream effects of secondary stress processes (such as high cholesterol or blood pressure) (Maestripieri and Hoffman 2011).

In addition to continuous exposure to a stressor leading to chronic stress, the intermittent, repeated application of acute stressors over a long time-period can cumulatively induce tertiary stress processes. As such, 'chronic intermittent stressor exposure' may be used to refer to frequent exposure to acute stressors over a period of weeks to months, and the resulting state is referred to as *cumulative* stress (Ladewig 2000). If the interval between stressor exposures does not allow for a complete restoration of homeostasis, over time the effects may accumulate and lead to stimulation of tertiary stress processes (Lee *et al.* 2015) (Figure 1). Over time, this accumulation may also result in a higher homeostatic set-point, meaning that even when given enough time to recover from a single acute stressor exposure, the stress response will not fully resolve to the pre-stress baseline (Lee *et al.* 2015; Herman *et al.* 2016). In addition, cumulative stress may not cause a down-regulation of the HPI axis, meaning that the animal is still be able to mount physiological responses to additional acute stressors (Herman *et al.* 2016). In this way, cumulative stress can be considered to be the sum of stress that has accumulated over an extended time period, regardless of the duration of individual stressors (Ladewig 2000). This "cumulation" of stress may also be referred to in the literature as allostatic load (Lee *et al.* 2015; Schreck and Tort 2016; Samaras *et al.* 2018).

As well as the duration and pattern of stressor exposure, the intensity of the stressor can influence its biological effects. Increased stressor intensity enhances the

physiological stress response, leading to increased cortisol secretion (Herman *et al.* 2016). Increasing the intensity may involve, for example, decreasing the distance between the animal and the stressor (e.g. closer predator exposure), or increasing the level of the stressor (e.g. stronger electric shock, worse crowding) (Inoue *et al.* 1994; Gronquist and Berges 2013). An enhanced acute physiological stress response caused by increased stressor intensity takes longer to resolve (slower return to homeostasis). Therefore, cumulative stress is more likely to result from exposure to repeated intermittent stressors if higher intensity stressors are applied (Herman *et al.* 2016).

Laboratory animal housing and regular husbandry procedures affect animals for long periods, if not the entirety, of their lives and so have the potential to elicit chronic stress, cumulative stress or both. While both chronic and cumulative stress have detrimental effects on the animal's physical and welfare state, their effects on indicators used to assess welfare differ. For example, cortisol concentration returns to baseline levels when chronic stress develops (see 1.2.6 below) whereas under cumulative stress cortisol concentration remains elevated due to an increased homeostatic set-point (Lee *et al.* 2015). When evaluating stress for the purpose of animal welfare assessment, it is important to be able to confirm the presence of long-term stress and this requires identifying which type of stress has been induced. In this thesis, the term cumulative stress is used to clearly delineate the summative induction of tertiary stress processes *without* HPI downregulation from the chronic induction of tertiary stress processes *with* HPI downregulation (chronic stress). In order to better understand the effects of housing and husbandry on animal welfare, the selected indicator or indicators should respond predictably to cumulative and chronic stress.

1.2.5.5 Modulation of stress responses due to stressor predictability and controllability

As well as the features of the stressor itself, an animal's perception of its own ability to predict it's situation can influence it's stress response and any associated affective experience (e.g. fear) (Bassett and Buchanan-Smith 2007; Cerqueira *et al.* 2020). In humans, low predictability of a negatively perceived (e.g. fearful) stressor increases feelings of fear (Vansteenwegen *et al.* 2008; Oka *et al.* 2010). In fish, there is evidence of a stronger physiological stress response to low predictability. For example, European sea bass (*Dicentrarchus labrax*) provided with a visual cue that predicted stressor exposure had lower plasma cortisol post-exposure than fish not provided with a cue (Cerqueira *et al.* 2020). Similarly, Mozambique tilapia (*Oreochromis mossambicus*) provided with a visual cue that predicted exposure to a 30 minute confinement stressor had lower plasma free cortisol concentrations than fish exposed to the same stressor but without a predictive cue (Galhardo *et al.* 2011). In addition, increased predictability reduces anxiety-like behaviours (Cerqueira *et al.* 2020). Taken together, these physiological and behavioural findings suggest that fish exposed to an unpredictable stressor were more likely to experience fear or anxiety.

Similarly to predictability, low controllability leads to an increased physiological stress response. Controllability refers to an animal's ability to perform an appropriate behavioural response that decreases the intensity or duration of a stressor. Often this involves avoiding the stressor by moving away (flight) or ending the stressor by physically abolishing it (fight). Experimentally, animals can be trained to perform a desired behaviour to trigger the removal of an applied stressor, allowing exploration of the effects of controllability on the stress response. For example, Carpenter and Summers (2009) conditioned rainbow trout (*Oncorhynchus mykiss*) to expect exposure

to a social stressor when water inflow to their tank was turned off. When this cue was given after seven days of training, fish that had been provided with an escape route during conditioning had plasma cortisol concentrations similar to their pre-trial baseline, but fish that had not been able to escape the stressor had a marked increase in cortisol. Thus, in determining the effects of an experimental stress protocol, not only the intensity and duration of the stressor but also the predictability and controllability must be considered.

1.2.5.6 Modulation of stress responses due to repeated or prolonged exposure

With repeated exposure, the pattern, intensity, predictability and controllability of stressor exposure can lead to changes in the animal's response to individual stressors (Moberg and Mench 2000), which should be considered in the selection of protocols for elicitation of longer-term stress. In addition, with prolonged exposure to a continuous stressor physiological adaptation can lead to changes in the observed response. In particular, an animal can become sensitised or desensitised to a stressor, which can alter its subsequent responses. Sensitisation is an enhancement of the stress response after repeated exposure to a stressor, meaning that the stressor becomes more salient to the animal and it is more likely to mount an acute physiological response (e.g. a measurable spike in cortisol concentration) (Stam *et al.* 2000). On the other hand, desensitisation is a dampening of the stress response to the same stressor, meaning that the stressor becomes less salient and the animal is less likely to exhibit an acute or measurable physiological response on subsequent exposures.

Sensitisation and desensitisation can occur through both psychological and physiological processes. Psychological desensitisation is referred to as acclimation (or habituation). Acclimation occurs due to learning and memory processes that improve the animal's ability to predict a stressor and successfully select a behavioural response that controls its exposure (Stam *et al.* 2000). For example, when the intensity and duration of a stressor remain stable with each repetition, the predictability of the stressor improves and this can lead to acclimation (Galhardo and Oliveira 2009). In addition, the animal may learn to predict a stressor by identifying an environmental cue that reliably predicts the onset of exposure (as seen in the above studies by Cerqueira *et al.* 2020 and Galhardo *et al.* 2011). Thus, an event or situation that elicited a stress response on first exposure may cease to elicit a stress response after repeated exposures (acclimation).

In contrast, if stressors are presented in such a way that they remain unpredictable and uncontrollable, acclimation does not occur. Therefore, long-term experimental stress protocols should be designed to maintain unpredictability and uncontrollability. This can be achieved by applying stressors at changing times-of-day, changing the type of stressor presented for each exposure, or changing the process of stressor application (to minimise the risk of inadvertently providing a predictive cue).

Physiological sensitisation and desensitisation are referred to as up- or down-regulation of physiological responses, respectively. In mammals, physiological downregulation occurs due to feedback mechanisms in the HPA axis (Smith and Vale 2006). In fish, the neuroendocrine mechanisms of HPI downregulation have not

yet been demonstrated (Gorissen and Flik 2016). However, there is evidence for the occurrence of these processes in fish after prolonged, continuous stressor exposure. For example, brown trout (*Salmo trutta* L.) exposed to a prolonged crowding stressor showed an initial increase in plasma cortisol concentration but returned to basal cortisol levels after four weeks, despite continued stressor exposure (Pickering and Pottinger 1989). Therefore, HPI downregulation is considered to be a response to chronic stress.

In terms of measurement of longer-term stress, acclimation and HPI downregulation have the same overall effect: a smaller response to a repeated or prolonged stressor. However, in terms of welfare the effect is opposite. Acclimation is a process of "getting used to" a stressor, so that it is perceived as less stressful. This leads to improved affective state, as the animal becomes, for example, less fearful. On the other hand, HPI downregulation is a process of physiological exhaustion, meaning that the animal may still have the same negative affective experience, but can no longer mount a physiological response to the stressor. The interaction between psychological and physiological processes over time makes it challenging to assess longer-term stress using currently available indicators and highlights the need to carefully design and clearly understand the type of stress elicited by experimental protocols.

1.2.6 Cortisol and other HPI-activation signals as indicators of zebrafish welfare

Cortisol is the most commonly used indicator of zebrafish welfare. Activation of the HPI axis results in a measurable increase in plasma cortisol within five to ten minutes

(Molinero *et al.* 1997). Measurement of cortisol concentration may be used for longitudinal studies by sampling the same individuals before and after the application of a stressor, or in cross-section by sampling from a non-stressed control group and comparing to a stressed treatment group. Therefore, cortisol is a versatile and informative parameter of acute stress in fish.

As noted above, long-term HPI activation can lead to a down-regulation of the axis and thus cortisol secretion (Aerts *et al.* 2015; Sadoul and Geffroy 2019). This makes it more challenging to use circulating cortisol concentration as a marker of chronic stress. Often, researchers do so by evaluating the animal's cortisol response to an additional acute stressor applied after a period of long-term exposure to the putative stressor. If no change in cortisol concentration is observed, this is taken to indicate that the HPI axis has been down-regulated and chronic stress has occurred (van de Nieuwegiessen *et al.* 2008; Santos *et al.* 2010).

Whilst this method may be useful for confirming the presence of chronic stress, it may not be useful for assessing the occurrence of cumulative stress. In such cases, the animal may be exposed to frequent stressors over a long time period but not exhibit HPI down-regulation, i.e. they can still respond to an acute stressor challenge. Therefore, it is difficult to predict how cortisol concentration will change under cumulative stress. To demonstrate, if an applied stress regime is expected to cause chronic stress, but a cortisol response to an acute stressor occurs, this result cannot be used to differentiate between the possibility that the animal was not chronically stressed and that it experienced cumulative stress. This restricts the utility of cortisol as a biomarker of long-term stress.

In addition to cortisol concentration, other hormone concentrations can be measured to indicate HPI activation. These include the precursors CRF and ACTH, and cortisone, a metabolically inactive product of cortisol breakdown (Ellis *et al.* 2012). However, the actions of CRF and ACTH occur very quickly after stress initiation, so are subject to similar constraints as catecholamines. Cortisone is considered a less direct measure of HPI activation than cortisol and is subject to the same limitations as cortisol. Additionally, the expression and activity of various stress-related genes such as brain-derived neurotrophic factor (BDNF) and POMC gene may also be used as stress biomarkers (Pavlidis *et al.* 2015). However, the mechanisms of expression for these genes are often not well understood and there is little standardisation for the selection of appropriate markers (Chakravarty *et al.* 2013; Manuel *et al.* 2014). In addition, these indicators are subject to the same limitations as cortisol itself when assessing long-term stress, i.e. downregulation due to chronic but perhaps not cumulative stress.

1.2.7 Behavioural indicators of zebrafish welfare

Zebrafish welfare can also be assessed using measures of behaviour but like cortisol, these measures have limitations. Affective experiences motivate animals to perform complex, adaptive behaviours (Fraser *et al.* 1997). As a consequence, by systematically interpreting animal behaviour we can make inferences about the welfare state of the animal. The most common forms of behavioural assessment are the observation of spontaneous behaviour (i.e. behavioural ethograms), and interactive testing paradigms such as novel tank diving and preference tests. The benefits of behaviour measurement are that it is low-cost, non-invasive and can be

used for longitudinal studies (Dawkins 2003). However, behavioural responses to cumulative and chronic stress may not be predictable due to difficulties in interpretation caused by individual variability and context-specificity.

Interpretation of both spontaneous behaviour and the outcomes of interactive testing paradigms can be problematic. Monitoring zebrafish and identifying particular welfare-relevant behaviours in the environment or during the treatment of interest can be used to understand how different conditions influence welfare. However, there is often large individual variability in response to identical conditions (Tran and Gerlai 2013). In addition, analysing behaviour in a welfare context requires an understanding of what affects specific behaviours reflect. Although some anxiety-like behaviours have been identified in zebrafish, such as increased shoaling, recent research suggests that similar behaviours may reflect excitement or exploration (Graham *et al.* 2018b). Therefore, applying welfare-relevant meaning to zebrafish behaviour is currently challenging. In addition, many behaviours (such as escape behaviours) represent attempts to cope with a specific problem, making them highly context specific. This limits the comparison of behavioural responses in different environments.

Interactive testing paradigms are used to address some of these challenges but are also prone to interpretation problems. Preference tests have been used to assess environmental enrichment for zebrafish (Schroeder *et al.* 2014). However, like humans, animals tend to select immediate reward over long-term benefit, so preference tests do not provide information about how environmental enrichment affects zebrafish in the long-term (Ainslie 1975; Duncan 1978). In addition,

preference tests are always relative, as it cannot be determined whether an animal is choosing within a set of high value or low value options (Duncan 1978; Jensen and Pedersen 2008; Nicol et al. 2009). Similarly, while it is widely assumed that less time spent at the bottom of a novel tank (less acutely fearful) indicates better welfare, the relationship between acute neophobia and overall welfare state is not clear. To illustrate, a chronically fearful animal may develop learned helplessness and cease responding to additional fear-inducing stimuli, giving a similar result to a less fearful animal (Dalla *et al.* 2008). In addition, handler effects may confound results (Seferta *et al.* 2001; Forkman *et al.* 2007).

The above limitations make behavioural analyses poorly suited for assessing long-term stress. For example, an animal that stops performing a particular behaviour in response to a stressor may have become acclimated to the stressor or may have developed learned helplessness. Therefore, measurement of behaviour cannot be used to distinguish between recovery from stress and continued stress. In addition, although cumulative stress causes an increased physiological stress response, the intensity of a behaviour does not directly reflect the magnitude of the stress response (Mellor *et al.* 2000). This means that behaviour cannot be used to distinguish between cumulative and chronic stress, reinforcing the need for a novel welfare indicator that responds predictably to chronic and cumulative stress.

1.2.8 There is a need for an animal-based indicator of cumulative experience in fish

When investigating the welfare implications of long-term situations (e.g. housing environment or husbandry practices), the welfare indicators selected must reflect the

cumulative experience of the animal. Over a longer time period, animals may enter a state of chronic stress but they may also experience periods of recovery that contribute to their overall experience (Bateson 2015). Existing fish welfare indicators such as cortisol concentration and behaviour cannot account for this. Thus, a current area of investigation is developing an indicator, or suite of indicators, that can.

A promising avenue for the development of indices of cumulative experience appears to be markers of ageing (Bateson and Poirier 2019; Bradshaw 2019). In particular, hippocampal volume and telomere dynamics have been identified as potential indicators in humans (Bateson and Poirier 2019). However, fish do not have a hippocampus or homologous brain structure. Additionally, the analogous brain structure (the lateral telencephalic pallium (Portavella *et al.* 2004)) in fish has not yet been imaged in vivo. Thus, telomere dynamics is the best option for further investigation.

1.1 Telomere length as a potential welfare indicator

Telomeres are non-coding DNA repeat sequences $(TTAGGG)_n$ found on the ends of chromosomes (Olsson *et al.* 2018). The term telomere dynamics refers to changes in telomere length, structure, and maintenance by the telomerase enzyme and shelterin protein complex, in response to a broad range of biological processes.

1.1.3 Telomere function and maintenance

Telomeres are protective structures at the terminal ends of chromosomes that protect DNA coding regions during repair and replication (Bateson 2015). This is achieved

by forming a loop (called a T-loop) that distinguishes the terminal chromosome ends from a double-stranded DNA break, thus avoiding inappropriate "repair" (de Lange 2005; Shay and Wright 2019).

During DNA replication, at the end of the lagging strand the final Okazaki fragment cannot be produced as there is nowhere for the RNA primer to attach. Therefore, a small section of DNA is left off after every round of replication. This is known as the end-replication problem (for review see Wellinger 2014). Because of this, telomeres shorten with every round of cell division in a process called attrition. When telomere length reaches a critical limit (the Hayflick limit), the T-loop can no longer be formed (de Lange 2018). Beyond this point the DNA coding region is at risk, and a pathway is triggered to induce cellular senescence and prevent further division (Shay and Wright 2000). Cellular senescence is an arrest in cell growth and division that occurs when a cellular DNA damage response is initiated (Van Houcke *et al.* 2015). This mechanism prevents damage propagation and protects against cancer, but it is also a hallmark of the biological ageing process (Sikora *et al.* 2011; Childs *et al.* 2015; Van Houcke *et al.* 2015).

Several protective measures have developed to maintain telomere integrity and thus protect cells against coding region damage. The reverse transcriptase enzyme telomerase repairs telomeres to restore length. Thus, when telomerase expression increases, telomeres shorten at a slower rate (Chawla and Azzalin 2008). Telomerase does this by synthesising telomeric repeats and adding them to telomere 3' ends (Chawla and Azzalin 2008; Cifuentes-Rojas and Shippen 2012). In addition, shelterin is a protein complex made up of six subunits (TRF1, TRF2, POT1, TIN2,

TPP1, and Rap1) that functions to maintain the T-loop structure. Shelterin modulates telomerase access to telomeres (de Lange 2005; Barcenilla and Shippen 2019), and protects the chromosome by blocking the access of DNA damage response enzymes (Xin *et al.* 2008), thus preventing chromosomal end-to-end joining (Lazzerini-Denchi and Sfeir 2016) and the activation of the cellular senescence pathway (Ben-Porath and Weinberg 2005).

1.1.4 Biological significance of telomere dynamics

The rate of telomere attrition is correlated to lifespan, with long-lived species exhibiting lower attrition rates than short-lived species (Haussmann and Marchetto 2010). In fact, telomere attrition has been identified as a hallmark of the vertebrate ageing process (López-Otín *et al.* 2013; Van Houcke *et al.* 2015). An increase in telomere attrition indicates an older biological age and therefore a significant change in biological function (Bateson and Poirier 2019).

1.1.5 Telomeres are also sensitive to stress

Telomere attrition has traditionally been associated with aging due to its role in replicative senescence. However, recently the association between telomeres and stress has been scrutinized more closely. The relationship between telomere dynamics and stress has recently been used in ecological research to explore life-history trade-offs and natural selection in wild animals (Ingles and Deakin 2016). This has led to a broadening of focus from the role of telomeres in ageing to their role in maintaining homeostasis and fitness.

Although these studies have had a strong focus on evolutionary processes, they have provided evidence linking changes in telomere dynamics to exposure to biologically relevant stressors (Monaghan 2014; Nettle *et al.* 2015; Angelier *et al.* 2018). The evidence produced by these studies is the basis for the suggestion that telomere attrition may be useful as a welfare indicator (Bateson 2015). In particular, there has been strong interest in the role of oxidative stress and glucocorticoids in telomere maintenance (Haussmann and Marchetto 2010; Barnes *et al.* 2019). Improved understanding of how glucocorticoids influence oxidative balance, and telomeres themselves, has led to the suggestion that the HPI axis plays a large role in the effect of stress on telomeres.

Cellular oxidative stress accelerates telomere attrition, with effects on both telomeres themselves and on telomerase. The guanine triplets of telomeres are particularly sensitive to oxidative damage from ROS (Bateson 2015; Angelier *et al.* 2018), and the presence of shelterin inhibits DNA repair mechanisms that would otherwise combat oxidative stress (Haussmann and Marchetto 2010). Oxidative stress also decreases telomerase activity (Kurz *et al.* 2004) meaning that telomeres cannot be rebuilt efficiently under these conditions. Finally, an increase in unrepaired oxidative damage to telomeres is correlated with increased telomere loss during cell division (von Zglinicki 2002). Consequently, an increase in oxidative stress is associated with increased telomere attrition and therefore shorter telomeres.

Glucocorticoids impact telomere length by directly interacting with telomerase, and by influencing the oxidative balance of the cell. Firstly, they directly modulate telomere maintenance mechanisms such as telomerase-mediated lengthening. The

specific effects depend on the degree of stress exposure, acute or chronic (Monaghan 2014; Mundstock *et al.* 2015). Prolonged exposure to elevated glucocorticoid levels results in the down-regulation of telomerase activity (Choi *et al.* 2008), but acute exposure may up-regulate it (Epel *et al.* 2010) leading to improved telomere rebuilding.

Secondly, glucocorticoids also mediate ROS production and antioxidant gene expression (Haussmann and Marchetto 2010; Costantini *et al.* 2011; Bateson 2015; Angelier *et al.* 2018). Chronic glucocorticoid secretion results in increased ROS and decreased antioxidant defences, leading to increased telomere damage and a greater attrition rate.

Finally, there is also the possibility that telomere dynamics can be affected even in the absence of ongoing high circulating levels of glucocorticoids, i.e. in the event of HPI-downregulation due to chronic stress. As noted above, tertiary stress leads to the dysregulation of the immune system. Immune dysregulation is associated with chronic low-grade inflammation, which causes increased cell turnover and oxidative stress (Bateson 2015; de Punder *et al.* 2019). Therefore, increased inflammation during chronic stress may also lead to an increased telomere attrition rate. This supports the idea that telomere dynamics may provide a useful indicator of both cumulative and chronic stress.

1.1.6 Current knowledge of telomere dynamics in fish

Zebrafish are a common model in gerontology (the study of ageing), a field that is closely invested in the study of telomere dynamics (Van Houcke *et al.* 2015). Because

zebrafish telomeres are similar in length to human telomeres (~5-15kb), considerable effort has been put into studying and characterising telomere dynamics in this species (McChesney *et al.* 2005; Carneiro *et al.* 2016a). The TTAGGG sequence found in humans and other vertebrates is conserved in zebrafish, and telomerase is expressed constitutively in somatic tissue throughout the lifespan (Anchelin *et al.* 2011). This means that, unlike humans, zebrafish telomeres can be rebuilt throughout their lives. In addition, all six shelterin subunits have been identified in this species (Xie *et al.* 2011; Wagner *et al.* 2017). The detailed characterisation of zebrafish telomeres, telomerase and shelterin means that these potential welfare indicators can be measured without requiring in-depth methodological validation beforehand.

The link between telomere dynamics and ageing in fish has been explored in multiple fish species, including zebrafish. In addition, the relationship between telomeres and *chronic* stress has been explored in commercially important fish species. For example Siberian sturgeon (*Acipenser baerii*) exposed to heat stress continuously for one month had telomeres on average 15% shorter than controls, suggesting that at least some forms of chronic stress cause marked telomere shortening in this species (Simide *et al.* 2016).

While it is not yet clear how conserved telomere dynamics are across different fish species, a recent meta-analysis found a significant correlation across all vertebrate taxa between stressor exposure and either a decrease in telomere length or an increase in telomere attrition (Chatelain *et al.* 2020). This analysis included nine studies on various species of fish, suggesting that the relationship between chronic stress exposure and telomere dynamics is evolutionarily conserved in fish. However, to the

best of my knowledge, no study has previously investigated the response of telomeres to *cumulative* stress in fish.

1.2 Research aims and objectives

The aim of this study was to investigate the effect of cumulative stress on telomere dynamics in zebrafish, in order to establish the usefulness of telomere dynamics as an indicator of cumulative welfare-relevant experience in this species. To achieve this, I conducted an experiment exposing zebrafish to an unpredictable chronic stress (UCS) regime and compared the length of their telomeres to those of non-stressed control zebrafish. In order to confirm that my experimental treatment induced a cumulative stress response, I also compared the cortisol response of the two groups. I hypothesised that fish exposed to the UCS regime would have shorter telomeres and higher whole-body cortisol concentration than non-stressed controls.

2 Methods

2.1 Ethics statement

Approval for the study procedures was granted by the Massey University Animal Ethics Committee (MUAEC Protocol 18/101). All procedures were carried out in accordance with the Massey University Code of Ethical Conduct for the Use of Animals for Research, Testing, and Teaching.

2.2 Animals and housing

A total of 121 AB/pet-shop (AB-PS) line wildtype zebrafish (*Danio rerio*) were sourced from the Otago Zebrafish Facility in Dunedin, New Zealand. Of these, 114 fish (72 males, 42 females) were used in the experiment and seven fish were used to train personnel to dissect out brains. Sex was determined visually based on body shape and colour (Yossa *et al.* 2013). Experimental fish were divided into control (n=57) and treatment (n=57) groups.

Within each of these groups, fish were allocated to sub-groups depending on what tissues would be sampled from them. Seventy-six fish (n=38 control and 38 treatment) were allocated for whole-body sampling, and 38 (n=19 control and 19 treatment) fish were allocated for whole-brain sampling. Fish used for whole-body sampling were further allocated to sub-groups to be used for either telomere length analysis (n=19 control and 19 treatment) or cortisol analysis (n=19 control and 19 treatment). These group sizes were determined using a power analysis based upon the results of a previous study investigating the cortisol response of zebrafish to an Unpredictable Chronic Stress (UCS) protocol similar to the one used here

($\mu1$=0.0015 ng/g, $\mu2$=0.0033 ng/g, Σ=0.002, α=0.05, β=0.80) (Song *et al.* 2018). The results of this analysis indicated that 16 fish per group would allow detection of the effect of the UCS treatment on whole-body cortisol. Extra fish were included to allow for any losses, and to allow for the same sex ratio to be maintained across all tanks.

All fish were selected from the same genetic line and hatched and raised in the same rearing tank. Fish were 13 months of age when they arrived, and 14 months at the start date of the experimental protocol.

2.2.3 Housing

Experimental fish were semi-randomly allocated to one of six 3.5 L tanks (approximately 17H x 10W x 26L cm) (Tecniplast, Milan, Italy) (n=19 per tank) at a sex ratio of 7 females:12 males, based on the numbers supplied. Three tanks held non-stressed controls (n=57) and three held fish exposed to the UCS treatment (n=57). Non-experimental fish were kept in a separate tank. Tanks were kept side-by-side on a SENTINEL rack (Tecniplast, Milan, Italy) on a recirculating water system (approx. 40 L) (Figure 1). Control tanks and UCS tanks were kept on separate water supplies to prevent sharing of any chemical communication molecules that may have passed through the filters and influenced the controls (e.g. free cortisol). Control fish were also visually isolated from UCS fish using opaque dividers between tanks.

Figure 1. Arrangement of experimental tanks on SENTINEL rack with two separate re-circulating water systems for control and treatment tanks: 2 sumps (black) and filtration systems.

Fish were maintained in standard laboratory conditions with a water temperature of 27-28°C, pH 7-8 and salinity of 0.25-0.75 ppt (200-1000 µS). A light:dark cycle of 14:10 h was used with light intensity increasing or decreasing over half an hour to simulate sunrise and sunset (Table 1) (Lawrence 2007; Reed and Jennings 2011; Avdesh *et al.* 2012). Ammonia, nitrite and nitrate levels were measured daily during fish introduction and habituation to monitor the response to loading the biological filter, then weekly during the stress protocol. If any levels were found to be outside of acceptable limits, a 25% water change was performed, and the water was re-tested to confirm that these levels were back within a normal range.

2.3 Fish maintenance

2.3.1 Habituation

Fish were initially habituated to their home tanks for two weeks (Piato *et al.* 2011; Song *et al.* 2018). During this time, both water systems were dosed daily with Stability® (Seachem Laboratories, GA, USA), a tank stabilisation solution containing nitrifying bacteria to support the biological filter. However, on day seven of habituation, nitrite levels spiked to 1 ppm, indicating that the biological filter was challenged (Lawrence 2007). This spike was managed with twice daily 25% water changes to lower the nitrite concentration and a 5 mL dose of Prime® (Seachem Laboratories, GA, USA) every 48 hours to detoxify nitrite while the nitrifying bacteria population in the biological filter re-established. The nitrite spike lasted three days. Due to the potential for this spike to have induced a physiological stress response in the fish (Carballo *et al.* 1995), the habituation period was extended by one week. Thus, fish were re-habituated to stable water parameters for two weeks prior to the start of the stress protocol.

2.3.2 Feeding and health monitoring

Fish were fed twice daily with ZM-400 pellets (Zebrafish Management Ltd., Winchester, UK), and once daily with freshly hatched *Artemia spp.* (ZM Brine Shrimp Cysts 230 Grade, Zebrafish Management Ltd., Winchester, UK) (Table 1). At each feeding time, all fish were checked for signs of injury or sickness (Table 2). Any fish that appeared injured or sick were removed from the home tank and isolated in a 1 L breeding tank (Tecniplast, Milan, Italy) for monitoring. If the injury or sickness was severe or there was no improvement after 24 hours, the fish was euthanised using rapid chilling at 2°C. Water changes (10 L, 25%) were performed for both

recirculating systems every second day. Complete standard operating procedures are available in Appendix A.

Table 1. Daily fish maintenance and treatment schedule. Morning and afternoon stressor treatments were performed daily at different times within the indicated time blocks.

Time of day		Action
AM	**6.00**	Light intensity begins increasing
	6.30	Lights at 100%
	7.00	Pellet feed
	7.30	
	8.00	
	8.30	
	9.00	
	9.30	AM treatment
	10.00	
	10.30	
	11.00	
	11.30	
PM	**12.00**	
	12.30	
	1.00	Live feed
	1.30	
	2.00	
	2.30	
	3.00	PM treatment
	3.30	
	4.00	
	4.30	
	5.00	
	5.30	
	6.00	Water change (25%, every 2nd day)
	6.30	
	7.00	Pellet feed
	7.30	Light intensity begins decreasing
	8.00	Lights off

Table 2. Signs of ill health for zebrafish, used for daily monitoring of fish.

Behavioural Changes	**Physical Changes**
Fish at surface	Colour change
Rapid breathing/gaping	Weight loss
Lethargy	Exophthalmia/Pop-eyes
Circling, twirling	Distended abdomen
Loss of equilibrium	Skeletal deformity
Rubbing on surfaces	Masses/swellings
	Haemorrhage/redness
	Gas bubbles
	Protruding scales
	Fin erosion or lesion
	Skin ulceration

2.4 Stress protocol

After the three-week habituation period, the UCS group were exposed to a battery of stressors for a four-week period. During this period the control fish were kept under the original conditions to which they were habituated. The chronic intermittent stress protocol selected to induce cumulative stress was modified from a UCS battery validated for zebrafish (Piato *et al.* 2011; Pavlidis *et al.* 2015; Song *et al.* 2018). Briefly, the main modifications to the published battery were the removal of the predator and zebrafish alarm substance exposure stressors as these treatments were not feasible to apply in our lab. Any social stressors such as isolation or group mixing were also removed. This was necessary as the fish were permanently sorted into tanks with identical sex ratios, and any mixing between tanks (intentional in the case of mixing or potentially accidental in the case of isolation) would confound a possible tank effect on cortisol levels or telomere length. In addition, any changes to social groups were likely to cause stress for an indeterminate time period after the end of the desired stressor application as social relationships were re-established. Finally, the protocol

was applied for a total duration of four weeks, compared to the one or two most often used in the literature.

Each day, two different stressors were applied to the three tanks of fish in the UCS treatment, with both the stressor and time of application varied each day to prevent desensitisation (see Appendix B) (Reed and Jennings 2011; Graham *et al.* 2018a). Six stressors that mimic typical husbandry procedures or environmental challenges were selected: chasing with a net, crowding, increased water temperature, decreased water temperature, dorsal body surface exposure due to low water level, and air exposure (Table 3).

2.3 Sampling

2.3.1 Euthanasia

At the conclusion of the four-week UCS protocol, all fish were euthanised using rapid chilling at 2°C as described below (Wilson *et al.* 2009; AVMA 2013). Sampling was performed in three rounds at approximately 1.20 pm, 2.09 pm, and 2.48 pm, the day after the final stressor was applied (Marcon *et al.* 2018b; Song *et al.* 2018). In each round, one control tank and one UCS tank were sampled simultaneously as follows (Figure 2.2). For each tank, all fish were netted together from their home tank and moved quickly to a 2°C ice slurry in a 1L tank. A two-minute timer was started when opercular movement ceased for all fish (Wilson *et al.* 2009). At the end of two minutes, six fish from each tank were moved to separate 1L tanks of ice slurry (separate for UCS and control). These tanks were moved to the dissection area for removal of the fish's brains. Because the brains were being stored for RNA analysis,

Table 3. Description of stressors selected for the UCS protocol and applied to zebrafish twice per day for four weeks.

Stressor	Description	Key references
Chasing	Chasing the fish in the home tank with a small net for a period of 5 minutes.	Manuel et al. 2014; Pavlidis et al. 2015
Crowding	Placement of a central partition in the home tank (halving the tank size) to effectively double the stocking density (10.8 fish/L) for a period of 50 minutes.	Ramsay et al. 2006; Pavlidis et al. 2015
Heating	Transferring fish from the home tank into a tank of system water pre-heated to 33°C for a period of 30 minutes then transferring back to the home tank.	Piato et al. 2011; Chakravarty et al. 2013; Manuel et al. 2014; Marcon et al. 2016
Cooling	Transferring fish from the home tank into a tank of system water pre-cooled to 23°C for a period of 30 minutes then transferring back to the home tank.	Piato et al. 2011; Chakravarty et al. 2013; Manuel et al. 2014; Marcon et al. 2016
Low water	Removal of water from the home tank until the dorsal body wall was exposed for a period of two minutes, then refilling with system water.	Piato et al. 2011; Chakravarty et al. 2013; Marcon et al. 2016; Rambo et al. 2017
Air exposure	Netting all fish in the tank and holding out of the water for a period of one minute. Performed a total of three times with ten minutes for recovery between repetitions.	Ramsay et al. 2009; Pavlidis et al. 2015; Fulcher et al. 2017; Song et al. 2018

it was important to remove and freeze the brains as quickly as possible to inactivate endogenous ribonucleases that could decrease RNA integrity (LoCoco *et al.* 2020).

Accurately sexing fish is difficult to achieve macroscopically, and visually assessing fish sex is a subjective and less reliable process (Yossa *et al.* 2013). Therefore, no attempt was made to balance the sex of fish selected for brain-removal as the extra time taken may have compromised sample quality. The remaining 13 fish per tank were retained for whole-body sample preparation.

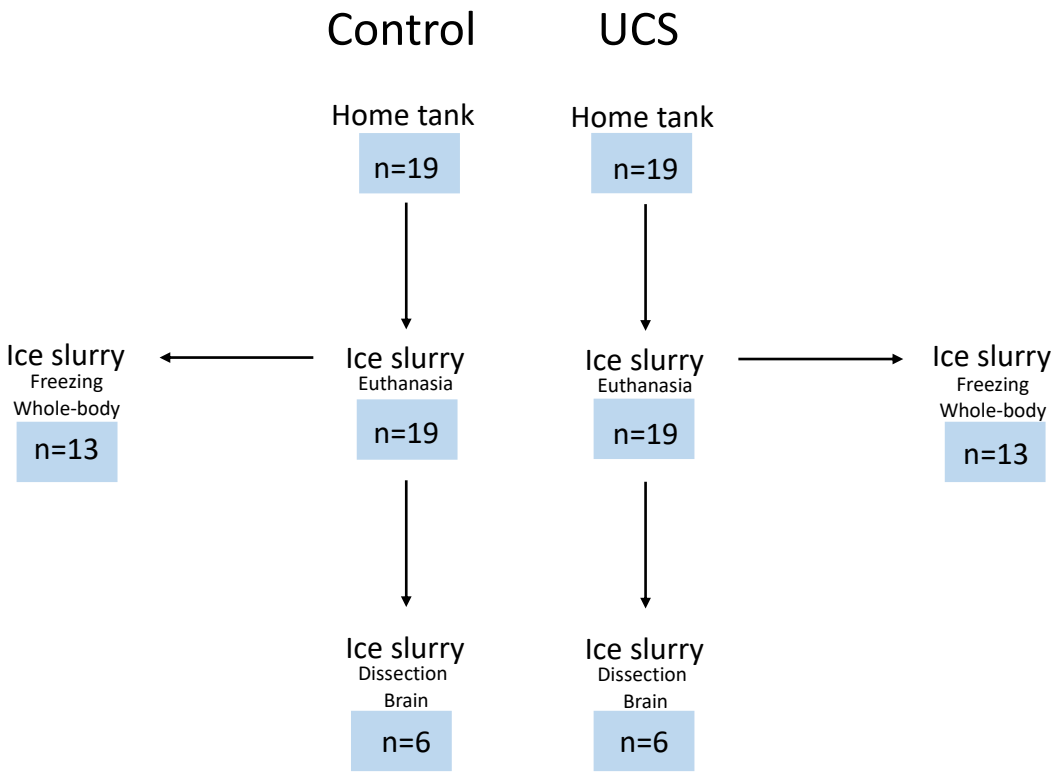

Figure 2. Sorting procedure for terminal sampling of all fish in one tank per treatment (control and UCS) at the conclusion of the four-week UCS protocol. This procedure was repeated three times at different time-points on the day after the final stress treatment. The numbers presented here represent the planned numbers, not accounting for losses.

2.3.2 Brain samples

Fish were removed individually from the ice slurry and their whole brains were dissected out under light microscopy (see Appendix C). Brains were transferred to individually labelled 2 mL Cryo.s™ freezing tubes (Greiner Bio-one, Kremsmünster, Austria) and immediately snap-frozen in liquid nitrogen. These samples were stored for future analysis of telomerase and shelterin activity, which was outside the scope of this thesis. The remains of the fish were discarded as damage sustained during dissection precluded them from further use.

2.3.3 Whole-body samples

Whole-body samples were prepared for cortisol and telomere analysis. The fish were removed from the ice slurry, blotted dry on a paper towel, placed whole into individually labelled 2 mL Cryo.s™ freezing tubes (Greiner Bio-one, Kremsmünster, Austria) and immediately transferred to a -80°C freezer for storage.

Table 4. Final number, type, and purpose of samples collected from the 114 experimental fish. Overall, there were eight losses. Four fish were lost due to injury during the habituation period (two found dead, two euthanised), one was used in a DNA extraction trial, and one was used in a cortisol extraction trial. In addition, two fish from the cortisol group were not sampled, resulting in a lower sample number than planned. In both the control and UCS treatment groups, the sex ratio was 31F:36M.

Sample type	Analysis	Control	UCS	Total
Whole Body	Telomere length	18	18	36
	Cortisol	17	17	34
Whole Brain		18	18	36
Losses		4	4	8
		57	57	114

2.4 Telomere analysis

Thirty-six whole-body samples (n=18 each of control and UCS) were stored at -80°C for five weeks before telomere analysis (Table 4). Before analysis, all frozen samples were weighed and sexed (Avdesh *et al.* 2012). Genetic analysis was performed at the Hopkirk Institute for Molecular Epidemiology and Public Health Laboratory, Palmerston North NZ.

2.4.1 Sample preparation

Samples were thawed at room temperature and descaled from the posterior base of the dorsal fin to the base of the caudal fin. The caudal fin was removed and two 1–2mm cross-sectional slices were taken from the peduncle to increase the surface area for digestion. Each of the slices contained a mixed proportion of muscle, skin, bone, and nervous tissue. The rest of the body and tail fin were returned to the tube and refrozen at -80°C.

2.4.2 DNA extraction

Genomic DNA was extracted from the tissue samples using a phenol-chloroform extraction protocol (Raschenberger *et al.* 2016). Both tissue slices from each fish were added to a 1.5 mL microcentrifuge tube with 150µL of a modified SDS lysis buffer (100 mM Tris-HCl, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) and 3.75 µL of Proteinase K 20 mg/mL (ThermoFisher, Waltham, MA, USA), and incubated for one hour at 56°C. The tubes were then vortexed to break up any remaining intact tissue and left to cool to room temperature. A 200 µL volume of equilibrated phenol:chloroform:iso-amyl alcohol (Sigma, St. Louis, MO, USA) was added and

the tubes were vortexed for a maximum of 10 seconds to homogenise, then centrifuged at 15000 rpm for 10 minutes at 4°C. The supernatant was then pipetted off into 1.5 mL centrifuge tubes containing a 10:1 100% ethanol:3M sodium acetate (Sigma, St. Louis, MO, USA) solution. This solution was stored at -20°C overnight.

The following day, the sample was centrifuged at 15000 rpm for 10 minutes at 4°C and the supernatant poured off. To clean the sample and precipitate the DNA, 200 μL of 70% ethanol was added, and the sample was centrifuged at 15000 rpm for five minutes at 4°C. The supernatant was then poured off and the cleaning process repeated. After pouring off the supernatant again, the tubes containing cleaned DNA were left open in a fume hood overnight to air dry. Once dry, DNA was resuspended in 50 μL of elution buffer (10 mM Tris-HCl pH 8.0).

2.4.3 Quantitative PCR and high-resolution melt analysis

A monoplex high-resolution melt (HRM) analysis real-time qPCR protocol was developed to amplify telomeric repeats and single-copy gene zebrafish *β-actin*.

Primers were obtained from Integrated DNA Technologies Inc. (IDT, IA, USA). The primers used for *β-actin* were ZF_actb_F (5'-CGA GCA GGA GAT GGG AAC C-3'), and ZF_actb_R (5'-CAA CGG AAA CGC TCA TTG-3') (McCurley and Callard 2008), and for telomeric repeats were telg (5'-ACA CTA AGG TTT GGG TTT GGG TTT GGG TTT GGG TTA GTG-3'), and telc (5'-TGT TAG GTA TCC CTA TCC CTA TCC CTA TCC CTA ACA-3') (Cawthon 2009). Primers were resuspended to 100 mM in appropriate volumes of nuclease-free water. The

following conditions were set up manually for the protocol: 1x HOT FIREpol® EvaGreen® HRM Mix (no ROX) (Solis Biodyne, Tartu, Estonia), 0.4 μL forward primer (0.2 μM), 0.4 μL reverse primer (0.2μM), 1 μL of DNA template, and MilliQ water to reach a final volume of 20 μL.

The qPCR and HRM were performed in a Rotor-Gene Q real-time PCR cycler (QIAGEN, Hilden, Germany) under the following conditions: activation cycle at 95°C for 12 minutes, followed by 40 cycles at 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Data were acquired at the elongation step at 72°C. HRM analysis was preconditioned at 72°C for 90 seconds and ramped to 95°C with a five second hold at each step. The final read-out provided a Cq value, one to four melt peaks, and an amplification factor for each well (one well for each target molecule per sample).

All samples were analysed in triplicate, with MilliQ water as a negative control. For each sample, both telomeric repeats and *β-actin* were analysed in separate wells of the same run, ensuring that PCR conditions were comparable for both assays. In addition, all samples from fish euthanised at the same time point were analysed in the same run to reduce potential variability.

2.4.4 Justification of telomere length measurement method

There are currently a number of different technical approaches to quantifying telomere length. These will be introduced here to explain and justify why monoplex qPCR was used.

The average length of telomeres can be quantified using either terminal restriction fragment (TRF) analysis, fluorescent in-situ hybridisation (FISH) or quantitative polymerase chain reaction (qPCR) techniques (Aviv *et al.* 2011; Gutierrez-Rodrigues *et al.* 2014; Lai *et al.* 2018). Each of these techniques has different advantages and limitations, but all require high-quality DNA samples and are strongly influenced by the DNA extraction process (Montpetit *et al.* 2014; Lin *et al.* 2019).

The current "gold standard" for telomere length measurement is terminal restriction fragment (TRF) analysis by Southern blot (Montpetit *et al.* 2014; Lai *et al.* 2018). This involves using a mix of restriction enzymes to digest the genomic DNA without cutting telomeric regions, then using agarose gel electrophoresis to separate DNA fragments by size. These fragments are then transferred to a filter membrane, exposed to radio-labelled probes that hybridise to the telomeric DNA, and viewed under x-ray to visualise the telomere fragments (Kimura *et al.* 2010). This technique provides a quantitative measurement of telomere length in kilobases, based on the distribution of bands on the gel (molecular weights of telomere fragments) and the fluorescence intensity of each band (Montpetit *et al.* 2014; Mender and Shay 2015).

However, depending on the mix of restriction enzymes selected, some genomic DNA adjacent to telomeres may not be digested, causing these sites to be included in the radio-labelled area and resulting in the over-estimation of telomere length (Montpetit *et al.* 2014; Lai *et al.* 2018). This makes it difficult to compare results between studies using different restriction enzymes but may be acceptable for comparative studies in which the absolute telomere length is less important than how telomere length

changes across time or treatments. In addition, TRF analysis requires large amounts of high-quality DNA, making it less suitable for some types of sample such as fixed tissue or samples that have been stored for a long time period (Montpetit *et al.* 2014). Finally, TRF analysis is a time-consuming process, making it less useful for large population studies compared to other available techniques (Gutierrez-Rodrigues *et al.* 2014; Lai *et al.* 2018).

FISH analysis involves hybridising a fluorescent peptide nucleic acid (PNA) probe to the telomeric sequences of all chromosomes within a cell (Montpetit *et al.* 2014). Measuring the average intensity of fluorescence from hybridised regions then can be used to indicate the quantity of telomere repeats in the cell (Gutierrez-Rodrigues *et al.* 2014). FISH allows the quantification of telomeres in different cell types within a sample, so can be used to explore the distribution of telomeres within cell populations (Aviv *et al.* 2011; Gutierrez-Rodrigues *et al.* 2014). However, FISH determines the average telomere content of a sample, expressed as average fluorescence intensity. This value can then be converted into kilobases based on its correlation with TRF analysis (Gutierrez-Rodrigues *et al.* 2014).

There are three different types of FISH analysis (interphase or metaphase quantitative FISH, and flow-FISH), but the details of each are outside the current scope (for review see Montpetit *et al.* 2014). FISH analysis is time-consuming to perform but is available commercially, making it a popular choice for larger-scale studies (Lai *et al.* 2018). However, PNA probes introduce some limitations. They are not able to hybridise to telomeres below a certain number of repeats, resulting in chromosome ends that appear to have no telomeres (O'Callaghan and Fenech 2011;

Montpetit *et al.* 2014; Lai *et al.* 2018). PNA probes may also bind to telomere repeats located in the genomic DNA (interstitial telomeric repeats), so may result in false positive results (Lai *et al.* 2018).

The final measurement technique for average telomere length is qPCR. This works by adding specific primers targeting telomere repeats and a fluorescent probe to the DNA sample and amplifying the telomere repeats over 20-40 heating cycles. The quantity of the PCR product (amplified telomeres) approximately doubles with each cycle, so by measuring the amount of fluorescence emitted the quantity of telomere repeats in the starting material can be calculated (Montpetit *et al.* 2014). Unlike TRF and FISH, qPCR does not require large amounts of starting DNA. In addition, qPCR is the most suitable technique for high-throughput research and large population studies (Gutierrez-Rodrigues *et al.* 2014). However, PCR is very sensitive to cross-contamination so must be performed with strict quality control (O'Callaghan and Fenech 2011).

Quantitative PCR can be utilised in three different ways to measure telomere length. The first, and most commonly used, qPCR technique quantifies telomeres by comparing the number of telomere repeats (T) to the number of repeats of a single-copy gene (S), producing the T/S ratio (Cawthon 2002). Initially, this was done by measuring the telomeres in one well and the single-copy gene in another well. This method is called monoplex qPCR. However, this may compromise the accuracy of the assay due to variation between wells (Montpetit *et al.* 2014). Thus, an updated method was developed to allow both telomeres and the single-copy gene to be

measured within the same well, using the same fluorescent dye (Cawthon 2009). This new method is called a monochrome multiplex qPCR.

The T/S ratio is not a direct measurement of average telomere length, rather it is a measure of the number of telomeric repeats relative to the number of repeats of the single-copy gene (Lin *et al.* 2019). It is useful for comparing results between treatment groups within a study, but is of limited use when comparing results between laboratories, particularly if different single-copy genes are used (Lai *et al.* 2018). In order to improve comparisons between laboratories, a qPCR method was developed for measuring absolute telomere length (O'Callaghan and Fenech 2011). This method uses serial dilutions of a synthesised standard of known size, containing only telomere repeats, to produce a standard curve. This standard curve can then be used to calibrate the PCR results and calculate the absolute length of telomeres in a sample. However, due to the potential for varying replicative histories in different cell types, absolute telomere length measurement is most accurate for samples made up of only a single cell type (O'Callaghan and Fenech 2011).

In the current research, the qPCR method was selected because TRF and FISH analyses were too time-consuming to be completed within the constraints of a Masters program. The monoplex method was chosen as this was the method that the research team was most familiar with. Finally, because a mixed tissue sample containing cells from muscle, skin, blood, and bone tissue was used, the absolute telomere measurement technique was not deemed appropriate. As this was a cross-sectional study comparing telomere lengths between two groups of animals, relative telomere length measurement was sufficient to address the study's hypothesis.

The single-copy gene selected was zebrafish *β-actin.* This gene was chosen because it has been previously validated as an appropriate single-copy gene for qPCR analysis using zebrafish tissues (McCurley and Callard 2008). In addition, *β-actin* has been previously used for qPCR analysis of telomerase in zebrafish (Henriques *et al.* 2013). Thus, it was selected for the telomere length assay presented here and for the planned future analysis of telomerase activity, allowing for more direct comparison between the two analyses.

2.5 Cortisol analysis

Thirty-four whole-body samples were stored for 19 weeks before cortisol analysis (Table 4). These samples were not weighed or sexed before analysis. Analysis was performed at the Hopkirk Institute for Molecular Epidemiology and Public Health Laboratory, Palmerston North NZ.

The cortisol concentration of fish can be measured in various mediums, including whole-body, plasma, faeces, and water (Ellis *et al.* 2012). The choice of sample type is influenced by the size of the animal being investigated. In large fish, cortisol may be measured in blood, but in small fish (such as zebrafish), it is usually taken as a whole-body measurement after terminal sampling (Sadoul and Geffroy 2019).

2.5.1 Cortisol extraction

Cortisol was extracted from frozen whole-body samples using a method adapted from Sink *et al.* (2007). This method has been used previously for analysis of zebrafish

cortisol responses to UCS (Piato *et al.* 2011; Rambo *et al.* 2017). Some changes were made to the published method due to equipment availability (see Discussion section).

Frozen samples were homogenised with 1 mL of phosphate buffered saline (PBS) in a bead beater for one minute then stored at -20°C overnight. The next day, 5 mL of diethyl ether was added and samples were centrifuged at room temperature for 10 minutes at 3000 rpm before re-freezing at -20°C overnight. The supernatant of diethyl ether containing cortisol was decanted off the frozen sample and the remaining tissue was discarded. The diethyl ether was then evaporated in a Savant SpeedVac Concentrator (SC210A, Thermo Fisher Scientific, MA, USA) for one hour at 65°C, leaving a lipid extract containing cortisol. This extract was stored at -20°C overnight.

2.5.2 Enzyme-linked immunosorbent assay analysis

Samples were thawed and resuspended in 60 μL PBS for enzyme-linked immunosorbent assay (ELISA) analysis. Analysis was performed with a Demeditec Cortisol ELISA DEH3388 Kit (Demeditec Diagnostics, Kiel, Germany). First, 10 μL of sample, calibrator, and control solutions were dispensed into appropriate wells, with samples added in triplicate. Then 200 μL of enzyme conjugate was added to all wells, and the plate was shaken for 10 seconds to mix, then incubated at room temperature for 60 minutes. After the incubation period the well contents were discarded and rinsed four times with 300 μL wash solution (diluted in 450mL MilliQ water, as per manufacturer instructions). Next, 200 μL of substrate solution was added to each well, and the plate was incubated in the dark without shaking for 30 minutes. After incubation, 50 μL of stop solution was added to each well.

The absorbance of each well was read at 450±10 nm within 15 minutes. ELISA plates were read in a VersaMax tunable microplate reader (Molecular Devices, CA, USA) with SoftMax Pro 5.4 software (Molecular Devices, CA, USA). Two ELISA plates were required to analyse all samples. All triplicates for each sample were analysed on a single plate.

2.6 Statistical analyses

Data were compiled in Microsoft Excel Version 16.31, and statistical analyses were performed in SAS (Statistical Analysis System University Edition 3.8, SAS Institute Inc., Cary, NC, USA). Significant differences between means were declared at $P<0.05$.

2.6.1 Relative telomere length

Telomere fold expression was calculated relative to *β-actin* expression from cycling threshold (Cq) values using the Pfaffl method (Pfaffl 2001), with the control average as the reference value. This method was selected due to variance in the amplification factors (a measure of the proportion of target molecules that are copied within one PCR cycle), as the other available model (the $2^{-\Delta\Delta Cq}$ method) assumes identical primer efficiencies for all samples (Livak and Schmittgen 2001; Svec *et al.* 2015). Because this study assessed relative, not absolute, telomere length, a standard curve was not generated (Lin *et al.* 2019).

The resulting data were analysed using the MIXED procedure to fit a mixed model that included the fixed effects of treatment (control and UCS), fish sex and weight (g), the interaction between treatment and sex, and the random effect of tank. Distribution of the data residuals was tested using the UNIVARIATE procedure and was found not to approximate normality. Therefore, comparisons of means were performed on the logarithm scale and presented after back-transformation, along with 95% confidence intervals.

2.6.2 Whole body cortisol

Standard curves were produced for each ELISA plate using absorbance (AU) and concentration (ng/ml) values from the six provided calibrator samples of known concentration. From these curves, cortisol concentration values were obtained for each well. The resulting data were analysed using the MIXED procedure to fit a mixed model that included the fixed effect of treatment (control and UCS) and the random effect of tank. Distribution of the data residuals was tested using the UNIVARIATE procedure and was found not to approximate normality. Comparisons of means were performed on the logarithm scale and presented after back-transformation, along with 95% confidence intervals.

3 Results

3.3 Health monitoring outcomes

During the project, four fish died unexpectedly. The first was found dead one day after the water parameters re-stabilised from the filter crash during the habituation period. It had no physical signs of injury or nitrite poisoning. The second was found during the habituation period twirling in the tank and was unable to swim straight – after removal from the tank a head injury was noticeable, so the fish was euthanised by rapid chilling. The third fish was found dead in the tank during the habituation period and showed signs of internal injury ventral to the operculum. The fourth fish was removed from its tank in the fourth week of the UCS protocol due to behavioural changes (lethargy, lying on the bottom of the tank) and a visibly distended abdomen indicative of being egg-bound. This fish was monitored in isolation for 24 hours but there was no improvement in its condition, so it was euthanised by rapid chilling.

3.4 Relative telomere length

A total of 36 whole-body samples were initially included in the telomere analysis. Of these, 29 were male and seven were female. In the control group, 14 fish were male and four were female while in the UCS group, 15 were male and three were female. A total of three samples ($n_{control}$=2, n_{UCS}=1) were excluded from the final analysis, because they could not be weighed.

Overall, 33 samples ($n_{control}$=16, n_{UCS}=17) were included in the final analysis. The back-transformed mean T/S ratio was 0.82 (95% CI 0.44-1.54) for the control group and 0.77 (95% CI 0.40-1.50) for the UCS group. The difference between means for

the control and UCS groups was not statistically significant (F=0.02, df=1,24, P=0.898).

A main effect of fish sex was found on the T/S ratio (F, df, P=0.002). Overall, female fish had a lower mean T/S ratio than did male fish (Female n=6: 0.52 (95% CI 0.29-0.94); Male n=27: 1.21 (95% CI 0.78-1.87)).

3.5 Whole body cortisol

A total of 34 whole-body samples ($n_{control}$=17, n_{UCS}=17) were included in the cortisol analysis. There was no effect of treatment on whole body cortisol concentration (F=0.07, df=1,28, P=0.787). The back-transformed mean cortisol concentration for the control group was 6.39 ng/mL (95% CI 4.35-9.39) and for the UCS group was 5.92 ng/mL (95% CI 4.03-8.70).

4 Discussion

Zebrafish are a popular model species in biomedical research, but little is known about how their housing conditions influence their welfare. Furthermore, currently available fish welfare indicators are inadequate for assessing long-term welfare because they do not respond predictably to chronic and cumulative stress. Telomeres have been suggested as a potential long-term welfare indicator due to clear links between chronic stress and telomere attrition in other vertebrate species.

The aim of this study was to investigate the use of telomere length as a biomarker of cumulative stress. I hypothesised that fish exposed to an UCS protocol for four weeks would have shorter relative telomere lengths than fish kept under standard husbandry conditions and not exposed to additional stressors. Contrary to my expectations, I found no effect of UCS on the T/S ratio of treated fish compared to non-stressed controls. However, I did find an effect of sex on T/S ratio. I also found no effect of UCS on whole-body cortisol concentration.

On the surface, these results appear to indicate that fish subjected to the UCS protocol were no more stressed than control fish. However, there are a number of different factors that might have influenced these findings, which need to be considered when drawing conclusions. The main reasons for these findings broadly relate to the possibility that the UCS protocol used did not sufficiently stress the fish, and the potential for stress in the control fish. These are discussed in detail below. In addition, I also discuss potential explanations for the observed sex effect on telomere length. Finally, important methodological factors are considered.

4.1 UCS protocol may have failed to induce sufficient stress

The failure to find a difference in relative telomere length could indicate that the cumulative stress regime imposed was not sufficiently stressful to elicit a change in telomere dynamics, as measured. This is supported by the failure to find an effect of treatment on whole-body cortisol. However, the UCS protocol used for this study was based on previous protocols that successfully elicited a cortisol response in zebrafish. Therefore, to explain why the protocol I used may not have produced the same response, it is useful to examine the differences between my UCS protocol and those previously published. The main differences were the duration of the protocol, the predictability of stressor application, and the intensity of the selected stressors. Each of these potential factors is considered here.

4.1.1 Protocol duration was not long enough to cause a measurable difference in telomere length

It is most probable that the duration of my protocol was not suitable to elicit cumulative stress or to elicit an effect of cumulative stress on telomeres. When selecting the duration, the two main factors to consider were making the protocol long enough to induce a measurable change in telomere length whilst keeping it short enough to mitigate the risk of stress desensitisation.

The response time of telomeres to cumulative stress has not previously been measured. In zebrafish, normal changes in telomere length have been investigated over different developmental periods, but none more closely spaced than two months (Anchelin *et al.* 2011). Therefore, it was not clear how long the UCS protocol should

be applied to induce a measurable telomere response. Consequently, the duration was selected based on knowledge from other vertebrate species.

In some species, telomeres respond to long-term continuous stressors in a timeframe of one to two weeks. For example, European starling chicks had shorter telomeres after nine days when raised with a competitive disadvantage (placed in a brood in which they were the smallest chicks) (Nettle *et al.* 2015). In addition, starling chicks placed on a restricted diet and stimulated to beg for food unsuccessfully had shorter telomeres after 15 days (Nettle *et al.* 2017). This suggests that a stress treatment lasting 9 days should be long enough to cause a measurable change in telomere length. However, the telomeres of juvenile vertebrates undergo rapid change during growth and development (Nettle *et al.* 2017) as a result of increased cell-turnover, so the effects of stress on telomere length may be magnified during this period. The fish used for this study were adults, so cell turnover rate, and thus telomere changes, were likely slower. This means that a longer time period might be required to detect a measurable change in telomere length.

In adult vertebrates, increased telomere attrition has been detected in response to long-term stress applied over two weeks, four weeks, eight months, and ten months (Ilmonen *et al.* 2008; Sohn *et al.* 2012; Cai *et al.* 2015; Pauliny *et al.* 2015). The broad range of these time periods may be reflective of the range of different stressors applied. The shortest of these experiments applied chronic crowding and food restriction to chickens for 14 days (Sohn *et al.* 2012). In contrast, the longest employed a transgenic line of salmon with an enhanced growth rate to investigate telomere attrition over ten months under the stress of rapid growth (Pauliny *et al.* 2015).

Both of these studies applied continuous stressors of different intensities, inducing *chronic* stress. However, my aim was to assess *cumulative* stress, i.e. the sum of stress caused by intermittent stressor application. Only one of the above studies applied a chronic intermittent stress protocol to elicit cumulative stress. Cai *et al.* (2015) used age-matched mice as a model for human depression. They applied each of five stressors (tail suspension, force-swim, foot shock, restraint, and sleep deprivation) once weekly for four weeks, after which stressed mice had shorter telomeres than non-stressed mice measured in saliva, blood, and liver tissue. This suggests that four weeks of chronic intermittent stress is long enough to cause a measurable difference in telomere length between treatments in adult vertebrates. I therefore chose to use a UCS duration of four weeks, in order to increase the likelihood of inducing a measurable change in telomere length. However, there is an important difference in the telomere dynamics of zebrafish and mice: telomerase.

4.1.1.1 Zebrafish telomeres may respond to stress more slowly due to the rebuilding actions of telomerase

Unlike mice and other mammals, adult fish express telomerase constitutively in somatic cells (Anchelin *et al.* 2011). Telomerase functions to lengthen telomeres after shortening, in order to maintain their integrity (Chawla and Azzalin 2008). This means that the fish in my study may have been rebuilding their telomeres during the UCS protocol. Under *chronic* stress, telomerase is inhibited and cannot efficiently rebuild telomeres. This is why previous studies in other fish species (e.g. Simide *et al.* 2016) have found an overall effect of telomere shortening in response to chronic stress, despite the presence of telomerase. Thus, it is likely that telomerase activity

differed between the control and UCS groups in this study, However, it is not clear how fish telomerase may respond to cumulative stress.

The response of telomerase to raised glucocorticoid levels is biphasic. Acute exposure to high levels of glucocorticoids may up-regulate telomerase activity, leading to repair and thus maintenance of telomere length in the short term. On the other hand, *chronic* glucocorticoid exposure inhibits telomerase activity, leading to impaired telomere maintenance and telomere shortening (Choi *et al.* 2008; Epel *et al.* 2010). Thus, under a 28-day *chronic* stress treatment I would expect to find telomere shortening. However, the UCS protocol for this study was not a continuous (chronic) stress treatment, but a chronic intermittent stress treatment designed to provide distinct periods of recovery between stressor applications. In this way, the stressors applied were acute, and may have stimulated repeated increases in telomerase activity.

The precise timing of the telomerase response is not clear (Epel *et al.* 2010). Elevation of telomerase has been found to occur between 12 hours and three days in human T and B lymphocytes in response to different stressors (Igarashi and Sakaguchi 1997; Hathcock *et al.* 2005). In contrast, after exposure to cortisol in vitro, telomerase activity remains constant for three days then drops below baseline levels (Choi *et al.* 2008). In my protocol, the time between afternoon and morning stressor applications was greater than 12 hours, so telomerase activity may have increased overnight. This could have either prevented telomeres from shortening at all or slowed the rate of shortening such that a significant difference in telomere length was not apparent within the 28-day timeframe. This would explain the failure to find an effect of UCS

on telomere length. Further research on telomerase activity in response to cumulative stress is required.

4.1.2 Protocol was too long and caused stress desensitisation

Another explanation for my finding of no difference in telomere length is that the fish may have desensitised to the UCS protocol due to its long duration. Desensitisation is a dampening of the stress response caused by either physiological downregulation or psychological acclimation.

The UCS protocol I applied was 28 days long. This is two to four times longer than most previous UCS protocols applied to zebrafish. Both seven (Piato *et al.* 2011; Manuel *et al.* 2014; Marcon *et al.* 2016; Rambo *et al.* 2017; Marcon *et al.* 2018a) and 14-day long UCS protocols (Piato *et al.* 2011; Manuel *et al.* 2014) induced a cortisol response, but my 28-day long protocol did not.

It was expected that the UCS protocol would elicit cumulative stress, meaning that whole body cortisol levels would still be higher in treated fish 24 hours after the application of the last stressor when the measurements were made. A final 'recovery period' of 24 hours should have allowed the acute stress response to the last stressor to resolve and cortisol levels to return to the current homeostatic set-point, which was expected to be higher in the UCS fish. In this case, telomere length and whole-body cortisol concentrations of the fish at time of euthanasia would have reflected the cumulative stress of the UCS protocol. However, the lack of difference in both cortisol concentration and telomere length between treatments suggests that any

acute stress response had resolved within 24 hours and that there was no cumulative stress.

In contrast, other studies that measured whole-body cortisol 24 hours after final stressor application found that UCS-exposed fish had higher concentrations than control fish (Piato *et al.* 2011; Marcon *et al.* 2016; Rambo *et al.* 2017). This suggests that either the acute HPI response of UCS-fish to the last stressor persisted 24 hours later or that their HPI homeostatic baseline (set-point) was elevated due to cumulative stress associated with the UCS protocol. The former option is unlikely as acute cortisol responses usually resolve within an hour (Dickerson and Kemeny 2004). In this case, the increased duration used for my protocol may have allowed for desensitisation (acclimation or downregulation) in my fish.

The UCS protocol used in my study may have caused chronic stress, leading to a downregulation of the HPI axis. However, telomere attrition rate is also influenced by inflammation and oxidative stress, independently of glucocorticoid secretion (Bateson 2015). Therefore, chronic stress would still be expected to cause a change in telomere length. Because of this, HPI downregulation is not the best available explanation for the failure to find a difference in cortisol concentration and telomere length between groups. In order to clarify whether HPI downregulation occurred, an acute stressor could be applied before euthanasia to determine whether a cortisol response could be mounted (e.g. Santos *et al.* 2010).

The longer duration of my protocol meant that fish were exposed to each stressor multiple times. Only one previous study has applied UCS to zebrafish for a time

period similar to the one I used. Song *et al.* (2018) used a 36-day protocol and found that exposed fish had a higher whole-body cortisol concentration than non-exposed control fish. This would suggest that acclimation was not caused by the increased duration. However, this study did not repeatedly present the same set of stressors but implemented a novel stressor every day, meaning that the stressors used were truly unpredictable. In contrast, with a repeating set of six stressors, the fish in my study were exposed to each stressor in the protocol at least eight times. Additionally, over the course of my protocol, although the six stressors were applied in changing order and time of day, they did not change in intensity or duration.

Because of the repetition of stressors, the fish may have learned to identify environmental cues that increased the predictability of the stressors, leading to psychological acclimation. In addition, the application of every stressor required removing the tank from the rack. Over time, this may have become a predictive cue for the fish that a stressor was about to be applied (Galhardo *et al.* 2011; Cerqueira *et al.* 2020). In addition, other cues such as the presence of timers or nets on the bench could have provided information about which stressor was about to be applied. Although they were not able to predict what time a stressor would be applied, the ability to predict an imminent stressor could have lowered the consequent stressfulness.

Even if the longer duration lead to a form of desensitisation in my study, previous studies suggest that a stress response may have occurred for at least the first 14 days of UCS application. In a mammalian species, the first 14 days of chronic intermittent stressor application would be expected to cause telomere attrition in the UCS-

exposed animals that was still measurable at the end of the protocol, as was seen when a chronic intermittent stress protocol was applied to mice for 4 weeks (Cai *et al.* 2015). However, as discussed above, adult zebrafish express telomerase in somatic tissue. Therefore, any telomere attrition caused by stress early in the protocol could have been repaired after desensitisation developed.

4.1.3 Stressor intensity was not strong enough

Although similar to UCS protocols used in previous zebrafish studies, the protocol I used excluded stressors that may have elicited particularly strong HPI-activation (i.e. predator exposure, alarm substance, or social stressors). In addition, the modifications made to some stressors, such as the lower crowding density, may have lowered their intensity and therefore made them less stressful. However, previous UCS protocols that successfully elicited an HPI response (as measured by cortisol) have varied greatly in their selection and application of stressor. For example, Piato *et al.* (2011) used heating, cooling, low water, chasing, crowding and tank change stressors, while Pavlidis *et al.* (2015) used lights off during the day or on during the night, chasing, net restraint, air exposure, and crowding. Neither of these studies used predator exposure, alarm substance, or social stressors. In fact, Pavlidis *et al.* (2015) used a potentially milder version of air exposure than the one in my protocol (1.5 minutes once compared to my one minute repeated three times) and used a similar crowding density (8 fish per litre compared to my 10.8). Therefore, it does not seem that the intensity of stressors used in my UCS protocol were dramatically different, and inadequate stressor intensity does not apparently explain the failure to find an effect of UCS on whole-body cortisol.

4.1.4 Summary

The evidence presented above strongly suggests that the failure to find a difference in telomere length between UCS-exposed and non-exposed fish can be explained by a failure to sufficiently induce cumulative stress in the UCS fish. This is likely to have occurred due to the duration and pattern of the UCS protocol applied. However, it is not clear whether the protocol was too short (not allowing for a telomere response in a species with constitutive telomerase activity) or too long (causing stress desensitisation and time to repair any initial telomere effect). This could be clarified in two ways: investigation into the response time of telomeres and the action of telomerase in response to cumulative stress; or by repeating the study using a different UCS protocol, by either implementing novel stressors every day or using a 14-day UCS protocol.

The following discussion considers alternative explanations under the assumption that cumulative stress was successfully induced in UCS fish.

4.2 Fish in the control group may have been chronically stressed

Assuming that the UCS protocol was effective in producing cumulative stress in treated fish, another potential explanation for the failure to find a difference in relative telomere length is that all of the fish (including controls) were chronically stressed by their housing conditions. Both control and UCS fish in my study were kept under standard conditions, as are currently used at the Otago Zebrafish Facility, and at the Western Australian Zebrafish Experimental Research Centre. They were kept at a density of 5.4 fish/L, more than twice that of any other study that used UCS

to induce stress in zebrafish. They were also kept in a relatively small space of only 3.5L, with a barren environment. Therefore, the housing conditions I used may have been considerably more stressful. This explanation is supported by the lack of difference found in cortisol between the two groups.

4.2.1 Effect of chronic stress on whole-body cortisol concentration

A high background level of HPI activation may have caused downregulation. This means that treated fish may not have been able to mount a stronger stress response to UCS, resulting in equal cortisol levels measured from both unexposed controls and UCS fish. Supporting this idea, zebrafish chronically housed at 4 fish/L (crowded) did not show any change in whole-body cortisol concentration in response to an acute and intense crowding stressor (40 fish/L) but those chronically housed at 0.2 fish/L (uncrowded) did (Ramsay *et al.* 2006). This suggests that the crowded zebrafish had a downregulation of the HPI axis, implying that they were chronically stressed.

One way to evaluate the likelihood of HPI downregulation is to compare the cortisol concentration values found here to those found in previous studies that have reported whole-body cortisol concentration in zebrafish subjected to an UCS protocol. However, because the fish sampled for whole-body cortisol in this study were not weighed before freezing, the resultant cortisol concentrations could not be expressed as concentration per gram of tissue, as others have done (Canavello *et al.* 2011). While this may limit the validity of comparing my results to those in the literature, a rough comparison may be made by normalising my cortisol concentrations to the average

body weight of the fish that were sampled for telomere length analysis (control average 0.483 g, UCS average 0.443 g).

Using this normalisation, control fish had an average whole-body cortisol concentration of approximately 13.2 ng/g of tissue, and UCS had an average concentration of approximately 13.4 ng/g of tissue. These values fall within the range of 10-15 ng/g of tissue reported for non-stressed control fish in other zebrafish UCS studies (Manuel *et al.* 2014; Marcon *et al.* 2016; Rambo *et al.* 2017). Assuming that this comparison is valid, this suggests that the fish in my study did not mount an additional stress response to the UCS protocol, supporting the explanation that they were chronically stressed.

However, there is surprising variance in the absolute cortisol concentration values reported in the literature, both for non-stressed and UCS fish. Piato *et al.* (2011) report approximately 1-3 ng/g of tissue for their non-stressed controls, whereas Marcon *et al.* (2018a) report approximately 20 ng/g of tissue. Despite a consistent trend for UCS fish to have higher cortisol concentrations than control fish, values reported for fish exposed to UCS are even more varied, ranging from 6.8 ng/g of tissue (Pavlidis *et al.* 2015) to approximately 60 ng/g of tissue (Marcon *et al.* 2018a). This wide variance among studies suggests that absolute cortisol values are less useful for comparative purposes than the relative values between treatments. Therefore, the numerical comparison presented above does not necessarily support chronic stress as a potential explanation for the failure to find an effect of UCS on whole-body cortisol.

The potential for chronic stress may have related to differences in the way the fish were kept in my study compared to previous studies. Although the studies discussed above consistently reported UCS-exposed fish having a higher whole-body cortisol concentration than non-exposed fish, none used standard zebrafish facilities to maintain their control and stressed subjects. These studies have used maintenance conditions that may have been less stressful than the conditions under which my fish were kept. For example, as mentioned above, the fish in my study were kept at a stocking density of 5.4 fish/L. In contrast, the zebrafish used by Piato *et al.* (2011) were kept at a stocking density of 1.3 fish/L. In addition, these fish were kept in 15L tanks, in contrast with my 3.5L tanks. As demonstrated above, a high stocking density may cause chronic stress in zebrafish (Ramsay *et al.* 2006). This supports the explanation that the fish in my study were stressed by their housing conditions and were not able to respond to UCS with stronger HPI activation due to downregulation.

4.2.2 Effect of chronic stress on telomere attrition

The close relationship between glucocorticoids and telomere dynamics could suggest that an effect on cortisol secretion caused by chronic stress would result in a similarly downregulated rate of telomere attrition. However, this is unlikely due to the potential for tertiary stress processes to influence telomeres independently of cortisol (Bateson 2015; de Punder *et al.* 2019). Instead, assuming that chronic stress was caused in both control and UCS fish by their housing conditions, the failure to find a difference in telomere length between groups supports the explanation that both groups had a similar rate of (likely increased) telomere attrition.

To further investigate whether chronic stress was stimulated in both control and UCS fish, it would be useful to confirm whether tertiary stress processes occurred in both groups. This could be achieved by measuring an indicator of immune dysregulation such as an inflammatory marker, or an indicator of oxidative stress such as ROS levels (de Punder *et al.* 2019).

4.3 Influence of fish sex on telomere response

Despite finding no effect of UCS on telomere length, there was an effect of sex. Female fish had shorter telomeres (lower T/S ratio) than male fish at the end of this study. This finding should be interpreted with caution because of the very small sample size of females (n=6) compared to males (n=27). In addition, fish were sexed while frozen. The most accurate way to sex zebrafish non-invasively is to identify the presence or absence of a genital papilla which is only present on females (Yossa *et al.* 2013). However, this is a very small structure that was not readily identifiable on frozen fish. Because this method was not feasible, fish were sexed based on a subjective judgement of their morphological characteristics, including body shape and colour. Sexing based on these features is a commonly used method, but its reliability has not been assessed (Yossa *et al.* 2013). Therefore, there was greater potential for error in identifying sex.

In zebrafish, sex difference in telomere length and telomere attrition have not previously been noted. However, most studies investigating telomere dynamics in fish either do not report sex (Horn et al. 2008; Hartmann et al. 2009; Lund et al. 2009; Henriques et al. 2013; Pauliny et al. 2015; Peterson et al. 2015), only include a single

sex (Carneiro *et al.* 2016b; Simide *et al.* 2016), or pool both sexes (Hatakeyama et al. 2008; Au et al. 2009; de Abechuco et al. 2016; Hatakeyama et al. 2016).

Assuming that the sex difference found in the present study is real, it may be due to inherent sexual dimorphism in telomere dynamics or stress responses in zebrafish, or due to greater stress in females due to the sex ratio under which fish in this study were kept. Supporting the existence of a sex effect on telomere length, sex differences in telomere length have also been found in mammals (Stindl 2004; Kotrschal *et al.* 2007; Ilmonen *et al.* 2008), birds (Hall *et al.* 2004; Salomons *et al.* 2009), reptiles (Ujvari and Madsen 2009; Olsson *et al.* 2010; Olsson *et al.* 2011), and one species of fish (Gopalakrishnan *et al.* 2013). These findings are not consistent for all species within these groups, reflecting important differences in species-specific biology. However, for the species in which sex-dependent differences in telomere dynamics were found, males consistently have shorter telomeres than females (Barrett and Richardson 2011). This observation is not yet well understood but has been linked to a wide range of biological factors including size dimorphism, heterogametic expression of telomere maintenance genes, and sex hormones (Stindl 2004; Barrett and Richardson 2011; Ingles and Deakin 2016).

Of particular interest is the suggestion the sexual size dimorphism could be an explanatory factor for sex differences in telomere length. A larger body requires more overall cell division during growth and for tissue maintenance in adulthood. Therefore, telomere attrition rates would be expected to be higher in the larger sex (Stindl 2004; Barrett and Richardson 2011). In zebrafish, females are larger than males (Ribas and Piferrer 2014) which is consistent with the idea of a higher rate of

telomere attrition and shorter telomeres in females, observed in the current study. However, in reptiles, in which females are the larger sex, males still have a higher rate of telomere attrition (Ujvari and Madsen 2009; Olsson *et al.* 2010).

Only one study has specifically investigated the effect of sex on telomere length in fish (Gopalakrishnan et al. 2013). Consistent with other vertebrate studies, this study found that female Japanese medaka (*Oryzias latipes*) had longer telomeres than males. This contrasts with my results which found that female zebrafish had shorter telomeres than males. However, there is no difference in body length of female and male medaka (Andrews 2005). Further research into the relationship between sexual dimorphism and telomere dynamics in zebrafish may elucidate whether body size is an important factor.

Another potential reason for the observed sex effect on telomere length is sexual differences in stress responses. In support of this idea, female zebrafish exposed to a 14-day UCS protocol had a smaller cortisol response to an acute stressor than did control females (Rambo *et al.* 2017). In contrast, UCS males showed a higher cortisol response than control males. The smaller response of females to the acute stress challenge suggests that they may have reached HPI exhaustion (chronic stress), implying that they had a stronger stress response to the UCS than males (Barcellos et al. 1999; Madaro et al. 2015).

Alternatively, shorter telomere length in females may have been caused by the social environment (i.e. biased sex ratio) in the tanks. The sex ratio used in the current study was strongly male-biased, with almost twice the number of males as females (7

female:12 male per tank), which may have caused females to experience greater stress due to chronic social conditions. Spence and Smith (2005) reported increased aggressive behaviour when zebrafish were kept in groups of 15 individuals with a male-biased sex ratio. In addition, zebrafish spawning behaviour involves chasing and other high-energy behaviours (Nasiadka and Clark 2012). In the male-biased social groups, females may have been targeted by multiple males during spawning, causing them to remain in a state of high arousal for extended periods of time. This supports the conclusion that females were likely to be under more stress than males, leading to shorter telomeres. In this study, the sex of fish sampled for whole-body cortisol concentration was not recorded. This information would have clarified the possibility for a sex effect on stress response.

Finally, it is possible that sex differences in the expression of the single-copy reference gene influenced the calculation of T/S ratio. In female zebrafish, three tissues (heart, skeletal muscle, and gonads) were found to have lower expression of *β-actin* than in males (McCurley and Callard 2008). A large proportion of the mixed tissue sample used for my study was skeletal muscle, meaning that the single-copy gene used to normalise the number of telomere repeats between samples may not have been constant between males and females. However, if this difference in *β-actin* expression between sexes occurred in the fish in this study, it would be expected to increase the T/S ratio of females. As the T/S ratio of females was significantly lower than that of males, the potential for a sex difference in *β-actin* expression cannot explain the sex difference in telomere length.

4.4 Methodological considerations

The most likely biological explanations for the observed results have been discussed above. However, it is important to address additional factors relating to the design of the study and measurement of outcome variables that may have influenced these results.

4.4.1 Cortisol assay reliability

For methodological reasons, it is not clear whether the reported cortisol concentrations accurately represented whole-body cortisol. Importantly, the process used for resuspending the lipid extract in preparation for the cortisol ELISA may have had significant impacts on the results. After acquisition of the lipid extract containing cortisol, this was resuspended in PBS solution as reported in previous zebrafish UCS studies (Piato *et al.* 2011; Manuel *et al.* 2014; Pavlidis *et al.* 2015; Marcon *et al.* 2016; Rambo *et al.* 2017; Marcon *et al.* 2018a). The protocol used by all of these studies can be traced back to a single study comparing resuspension methods for cortisol extracts from Golden Shiners (*Notemigonus crysoleucas*) (Sink *et al.* 2007). Interestingly, this study reported resuspension in PBS as failing to meet three of their validation criteria (precision of recovery from dilutions, linearity, and parallelism). As time constraints precluded me from testing and validating another resuspension technique (such as vegetable oil, as suggested by Sink *et al.* 2007), I chose to use a PBS resuspension in accordance with the zebrafish UCS literature. However, when vortexed in PBS, the lipid extract formed oil droplets in the suspension. It was not clear whether the volume of extract picked up by pipetting was standard across all samples, or whether the ratio of extract to PBS differed. In addition, the lipid extract

visibly adhered to the sides of the pipette tip and could not be uniformly ejected into the ELISA well.

To gauge the accuracy of the cortisol assay in the present study, the results could be compared with previous zebrafish UCS studies. However, as discussed above (section 4.2.1), the validity of comparison between studies is limited due to the wide variance of absolute whole-body cortisol values presented in the literature. Therefore, although the normalised average whole-body cortisol concentrations calculated in section 4.2.1 fell within the range of previously reported values, this does not provide sound supporting evidence for the reliability of the cortisol extraction process used here.

4.4.2 Telomere assessment

Two main methodological factors may have influenced the T/S ratio results, independent of the biological effects of the UCS protocol. Firstly, the use of a mixed tissue sample may have obscured changes in telomere length in specific tissues. Secondly, metrics indicating the quality of the DNA used to calculate T/S ratio were not measured.

A mixed tissue sample would have included various different tissue types, and telomere length and attrition rate are known to vary among tissues. So although the mixed tissue sample represents an average telomere length across tissue types, this average value would be influenced by the proportion of different tissues and their specific telomere dynamics (Carneiro *et al.* 2016b). In general, proliferative

tissues such as gut and gonadal tissue have higher telomere attrition than non- or low-proliferative tissues such as bone, due to increased cell turnover. An exception to this is muscle, a low proliferative tissue which, in zebrafish, has a high telomere attrition rate, potentially due to increased levels of oxidative stress (Carneiro *et al.* 2016b).

The decision to extract DNA from a mixed tissue sample was based on the difficulty of dissecting out a single tissue-type or organ from the frozen samples. The section removed from the fish for analysis consisted of mostly low proliferative tissues (muscle, bone, nervous tissue) and did not contain any visceral organ tissue. The samples also contained skin and bone marrow, two tissues with high proliferative capacity (Lee *et al.* 1998; Buckingham and Klingelhutz 2011). Although subjectively the samples appeared to have a high proportion of muscle tissue, the proportion of each tissue type was not quantified. Therefore, the lower turnover rates (and thus lower telomere attrition) of the low proliferative tissues may have masked changes that occurred in the high proliferative tissues and muscle. Had I selected a single tissue type, particularly a high proliferative tissue, I might have been more likely to find a change in telomere length within the 28-day protocol.

Due to constraints on equipment availability, the quality of the DNA used for telomere measure was not analysed. Specifically, the 260/280 ratio, which signifies DNA purity and can be used to identify RNA contamination, and the DNA concentration (yield) were not quantified for each sample. These measures are important to ensure that DNA extraction was successful and that the results of

subsequent analysis (in this case qPCR) accurately reflect the intended parameter (Koetsier and Cantor 2019; Lin *et al.* 2019). Thus, DNA quality may have influenced the telomere measurements reported here.

4.4.3 Low-influence factors

Finally, it is important to identify factors with the potential to influence results that were sufficiently accounted for and are therefore unlikely to contribute to the above explanations. Here I briefly note the measures that were taken in the study design to minimise the probability that any of these aspects could have influenced the results.

4.4.3.1 Study population

The study population was carefully selected to minimise variation in initial telomere length among individuals. Telomere length is heritable, and heavily influenced by the developmental environment (Angelier et al. 2018), so I used fish from the same generation of the same genetic line that were incubated and hatched at the same time and raised together in the same tank. Developmental change in telomere length seems to stabilise around 7 months of age, and age-related telomere attrition begins to occur after 18 months of age in zebrafish (Anchelin *et al.* 2011). Therefore, in addition to ensuring low variation in telomere length due to genetic and developmental conditions, I ensured that any changes in telomere length could be separated from age-related decline by using age-matched 14-month-old fish.

4.4.3.2 Housing and husbandry conditions

The experimental conditions were controlled as tightly as possible. All fish were maintained in the same temperature- and light-controlled room. They were also exposed to the same husbandry procedures; water changes were always performed on both systems at the same time, fish were fed the same food, and were always checked at the same time. The UCS fish were also visually and chemically isolated from control fish, to prevent any behavioural or chemical cues from inducing stress in controls.

4.4.3.3 Sampling procedure

Finally, the time elapsed between removing the fish tanks from the SENTINEL rack and their death may have influenced the observed cortisol concentrations. The disturbance of removing the tank and netting the fish is likely to have stimulated HPI activation, causing an acute change in circulating cortisol levels. However, the influence of this on the observed whole-body cortisol concentration could be mitigated by ensuring that the time between disturbance and death was minimized. In other fish species, a measurable change in cortisol concentration takes at least four minutes post-stressor initiation to develop (Lawrence *et al.* 2018). Overall, the time elapsed between removal from the rack and entering the ice-water bath was less than a minute. One of the advantages of rapid-chilling euthanasia for zebrafish is the short time to loss of opercular movement (less than 10 seconds), currently used to represent time of death (Wilson *et al.* 2009). In addition, the low temperature immediately slows the rate of biological processes. This minimised the likelihood of HPI stimulation culminating in increased cortisol levels.

4.5 Conclusions

The application of a UCS protocol did not result in a change in telomere length in the zebrafish in this study. In addition, no difference in whole-body cortisol concentration, measured 24 hours after the last stressor, was observed between UCS and control fish. Based on these results, it is not possible to conclude whether telomeres change under cumulative stress in this zebrafish model, as I was unable to demonstrate that the fish in this study were stressed.

The most likely reason for the failure to find an effect of UCS on cortisol and telomere length is that the protocol applied did not sufficiently cause cumulative stress. The protocol may have been too short to account for the rebuilding of telomeres by telomerase under cumulative stress, or it may have been too long, allowing the fish to acclimate to the stressors. In order to determine which of these explanations is most parsimonious, it would be useful to measure the activity of telomerase, or to assess the telomere response to a shorter UCS protocol (e.g. 14 days). It is also possible that the cortisol concentration and telomere length were not different between groups because both groups were equally (chronically) stressed. The measurement of tertiary stress processes in both groups would identify whether the fish were under chronic stress.

The research presented here highlights the difficulties in developing indicators that can predict chronic or cumulative stress in order to provide reliable information on longer-term welfare state. It demonstrates how cortisol alone cannot be used to distinguish between a failure to cause stress, or the induction of chronic stress. Although it could not be demonstrated here, telomere length may still be a valuable

welfare indicator but, as noted above, additional indicators are required to interpret how telomeres respond to long-term (both chronic and cumulative) stress.

Finally, this study is the first report of a sex-related difference in telomere length in zebrafish. It is not clear whether this reflects a sex-related difference in stress responsiveness to the UCS regime or the social environment or whether it is an inherent biological phenomenon in this species. Nonetheless, this finding provides important knowledge about zebrafish telomere dynamics that should be accounted for in future research.

4.6 Future directions

The results of this study point to a range of future directions for improving our understanding of stress and telomere dynamics. In particular, two routes of investigation stand out at useful next steps. These routes explore details surrounding two main questions:

1 How do different types and patterns of long-term stress influence telomere dynamics in zebrafish?

2 Is there a sex-dependent difference in zebrafish telomere dynamics?

To understand how long-term stress influences telomere dynamics, particularly in the context of developing telomere dynamics as an animal welfare indicator, future investigations first need to differentiate between cumulative and chronic stress. In order to understand whether telomeres respond to each of these types of stress in a predictable way, it must be clear which type of stress is being assessed. This can be

achieved by applying stressors continuously for the duration of a test to cause chronic stress, or by applying a chronic intermittent stress protocol to cause cumulative stress.

In order to better identify long-term stress, it would be useful measure tertiary stress markers such as reproductive output, growth rate, or disease morbidity (Sopinka *et al.* 2016). Once the occurrence of long-term stress has been confirmed, cortisol concentration could be used to differentiate between chronic and cumulative stress by applying an acute stress test before sampling. In this case, animals under chronic stress would not show a change in cortisol concentrations due to HPI downregulation, whereas those under cumulative stress would still be able to mount an acute response.

Telomere dynamics, including the response of telomerase and shelterin, should be characterised under both chronic and cumulative stress in order to determine whether their response can be reliably predicted under both conditions. If so, telomerase in particular may be another potential welfare indicator worthy of further investigation. In addition, part of the required characterisation of telomere dynamics includes the timeframe of response to stressor application. It is important to know how long it takes for changes in telomere attrition rate, and telomere length, to occur. This is likely to be closely linked to the response times of telomerase; although some literature exists in this area it would be useful to clarify how telomerase responds to stress in vivo in adult zebrafish.

Finally, it would be valuable to further explore the potential for a sex difference in the telomere dynamics of zebrafish. As an emerging model species for vertebrate

telomere dynamics, zebrafish are increasingly used to investigate the role of telomeres in human diseases such as cancer (Carneiro *et al.* 2016a). If a sex effect exists and is not identified, the application of knowledge gained from zebrafish will be limited.

5 References

Aerts J, Metz JR, Ampe B, Decostere A, Flik G, De Saeger S. Scales tell a story on the stress history of fish. *PLOS ONE* 10, e0123411-e, doi:10.1371/journal.pone.0123411, 2015

Ainslie G. Specious reward: A behavioral theory of impulsiveness and impulse control. *Psychological Bulletin* 82, 463-96, doi:10.1037/h0076860, 1975

Almeida O, Gozdowska M, Kulczykowska E, Oliveira RF. Brain levels of arginine–vasotocin and isotocin in dominant and subordinate males of a cichlid fish. *Hormones and Behavior* 61, 212-7, doi:https://doi.org/10.1016/j.yhbeh.2011.12.008, 2012

Anchelin M, Murcia L, Alcaraz-Pérez F, García-Navarro EM, Cayuela ML. Behaviour of telomere and telomerase during aging and regeneration in zebrafish. *PLOS ONE* 6, e16955, doi:10.1371/journal.pone.0016955, 2011

Andrews AL. The role of female preference in sexual dimorphism of Japanese medaka (*Oryzias latipes*). The Ohio State University, 2005

Angelier F, Costantini D, Blévin P, Chastel O. Do glucocorticoids mediate the link between environmental conditions and telomere dynamics in wild vertebrates? A review. *General and Comparative Endocrinology* 256, 99-111, doi:https://doi.org/10.1016/j.ygcen.2017.07.007, 2018

Animal Welfare Act, 1999. Retrieved 20 May, 2020 from http://www.legislation.govt.nz/act/public/1999/0142/59.0/DLM49664.html

Arends RJ, Mancera JM, Munoz JL, Wendelaar Bonga SE, Flik G. The stress response of the gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement. *Journal of Endocrinology* 163, 149-57, 1999

Arjona FJ, Vargas-Chacoff L, Ruiz-Jarabo I, Gonçalves O, Páscoa I, Martín del Río MP, Mancera JM. Tertiary stress responses in Senegalese sole (*Solea senegalensis* Kaup, 1858) to osmotic challenge: Implications for osmoregulation, energy metabolism and growth. *Aquaculture* 287, 419-26, doi:https://doi.org/10.1016/j.aquaculture.2008.10.047, 2009

Au DWT, Mok HOL, Elmore LW, Holt SE. Japanese medaka: A new vertebrate model for studying telomere and telomerase biology. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 149, 161-7, doi:https://doi.org/10.1016/j.cbpc.2008.08.005, 2009

Avdesh A, Chen M, Martin-Iverson MT, Mondal A, Ong D, Rainey-Smith S, Taddei K, Lardelli M, Groth DM, Verdile G, Martins RN. Regular care and maintenance of a zebrafish (*Danio rerio*) laboratory: an introduction. *Journal of Visualized Experiments : JoVE*, e4196-e, doi:10.3791/4196, 2012

Aviv A, Hunt SC, Lin J, Cao X, Kimura M, Blackburn E. Impartial comparative analysis of measurement of leukocyte telomere length/DNA content by Southern blots and qPCR. *Nucleic Acids Research* 39, e134-e, doi:10.1093/nar/gkr634, 2011

AVMA. AVMA guidelines for the euthanasia of animals: 2013 edition. *American Veterinary Medical Association*, 2013

Barcellos LJG, Nicolaiewsky S, De Souza SMG, Lulhier F. Plasmatic levels of cortisol in the response to acute stress in Nile tilapia, *Oreochromis niloticus* (L.), previously exposed to chronic stress. *Aquaculture Research* 30, 437-44, doi:10.1046/j.1365-2109.1999.00348.x, 1999

Barcellos LJG, Marqueze A, Trapp M, Quevedo RM, Ferreira D. The effects of fasting on cortisol, blood glucose and liver and muscle glycogen in adult jundiá Rhamdia quelen. *Aquaculture* 300, 231-6, doi:https://doi.org/10.1016/j.aquaculture.2010.01.013, 2010

Barcenilla BB, Shippen DE. Back to the future: The intimate and evolving connection between telomere-related factors and genotoxic stress. *Journal of Biological Chemistry* 294, 14803-13, 2019

Barnes RP, Fouquerel E, Opresko PL. The impact of oxidative DNA damage and stress on telomere homeostasis. *Mechanisms of Ageing and Development* 177, 37-45, doi:https://doi.org/10.1016/j.mad.2018.03.013, 2019

Barrett ELB, Richardson DS. Sex differences in telomeres and lifespan. *Aging Cell* 10, 913-21, doi:10.1111/j.1474-9726.2011.00741.x, 2011

Barton BA, Iwama GK. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases* 1, 3-26, doi:https://doi.org/10.1016/0959-8030(91)90019-G, 1991

Barton BA. Stress in fishes: A Diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology* 42, 517-25, doi:10.1093/icb/42.3.517, 2002

Bassett L, Buchanan-Smith HM. Effects of predictability on the welfare of captive animals. *Applied Animal Behaviour Science* 102, 223-45, doi:https://doi.org/10.1016/j.applanim.2006.05.029, 2007

Bateson M. Cumulative stress in research animals: Telomere attrition as a biomarker in a welfare context? *BioEssays* 38, 201-12, doi:10.1002/bies.201500127, 2015

Bateson M, Poirier C. Can biomarkers of biological age be used to assess cumulative lifetime experience? *Animal Welfare* 28, 41-56, doi:10.7120/09627286.28.1.041, 2019

Bayne K. Environmental enrichment and mouse models: Current perspectives. *Animal Models and Experimental Medicine* 1, 82-90, doi:10.1002/ame2.12015, 2018

Beausoleil NJ, Mellor DJ. Validating indicators of sheep welfare. In: *Achieving sustainable production of sheep*. 2017

Ben-Porath I, Weinberg RA. The signals and pathways activating cellular senescence. *The International Journal of Biochemistry & Cell Biology* 37, 961-76, doi:https://doi.org/10.1016/j.biocel.2004.10.013, 2005

Bernier NJ, Flik G, Klaren PHM. Chapter 6 Regulation and contribution of the corticotropic, melanotropic and thyrotropic axes to the stress response in fishes. In: *Fish Physiology*. Pp 235-311. Academic Press, 2009

Bradshaw W. Assessing biomarkers of ageing as measures of cumulative animal welfare. *EcoEvoRxiv,* doi:10.32942/osf.io/uj4mt, 2019

Braithwaite VA. Assessing fish welfare. *CAB Reviews* 12, 1-7, doi:10.1079/pavsnnr201712023, 2017

Branson EJ. *Fish Welfare.* Oxford ; Ames, Iowa : Blackwell Pub., 2008

Buckingham EM, Klingelhutz AJ. The role of telomeres in the ageing of human skin. *Experimental Dermatology* 20, 297-302, doi:10.1111/j.1600-0625.2010.01242.x, 2011

Bury NR, Sturm A. Evolution of the corticosteroid receptor signalling pathway in fish. *General and Comparative Endocrinology* 153, 47-56, doi:https://doi.org/10.1016/j.ygcen.2007.03.009, 2007

Cai N, Chang S, Li Y, Li Q, Hu J, Liang J, Song L, Kretzschmar W, Gan X, Nicod J, Rivera M, Deng H, Du B, Li K, Sang W, Gao J, Gao S, Ha B, Ho H-Y, Hu C, Hu J, Hu Z, Huang G, Jiang G, Jiang T, Jin W, Li G, Li K, Li Y, Li Y, Li Y, Lin Y-T, Liu L, Liu T, Liu Y, Liu Y, Lu Y, Lv L, Meng H, Qian P, Sang H, Shen J, Shi J, Sun J, Tao M, Wang G, Wang G, Wang J, Wang L, Wang X, Wang X, Yang H, Yang L, Yin Y, Zhang J, Zhang K, Sun N, Zhang W, Zhang X, Zhang Z, Zhong H, Breen G, Wang J, Marchini J, Chen Y, Xu Q, Xu X, Mott R, Huang G-J, Kendler K, Flint J. Molecular signatures of major depression. *Current Biology* 25, 1146-56, doi:https://doi.org/10.1016/j.cub.2015.03.008, 2015

Canadian Council on Animal Care. Animal Data Report. 2018

Canavello PR, Cachat JM, Beeson EC, Laffoon AL, Grimes C, Haymore WAM, Elegante MF, Bartels BK, Hart PC, Elkhayat SI, Tien DH, Mohnot S, Amri H, Kalueff AV. Measuring endocrine (cortisol) responses of zebrafish to stress. In: Kalueff AV, Cachat JM (eds). *Zebrafish Neurobehavioral Protocols*. Pp 135-42. Humana Press, Totowa, NJ, 2011

Carballo M, Munoz MJ, Cuellar M, Tarazona JV. Effects of waterborne copper, cyanide, ammonia, and nitrite on stress parameters and changes in susceptibility to saprolegniosis in rainbow trout (*Oncorhynchus mykiss*). *Applied and Environmental Microbiology* 61, 2108, 1995

Carneiro MC, de Castro IP, Ferreira MG. Telomeres in aging and disease: lessons from zebrafish. *Disease Models & Mechanisms* 9, 737, 2016a

Carneiro MC, Henriques CM, Nabais J, Ferreira T, Carvalho T, Ferreira MG. Short telomeres in key tissues initiate local and systemic aging in zebrafish. *PLOS Genetics* 12, e1005798, doi:10.1371/journal.pgen.1005798, 2016b

Carpenter RE, Summers CH. Learning strategies during fear conditioning. *Neurobiology of Learning and Memory* 91, 415-23, doi:https://doi.org/10.1016/j.nlm.2009.01.009, 2009

Cawthon RM. Telomere measurement by quantitative PCR. *Nucleic Acids Research* 30, e47-e, 2002

Cawthon RM. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. *Nucleic Acids Research* 37, e21-e, doi:10.1093/nar/gkn1027, 2009

Cerqueira M, Millot S, Felix A, Silva T, Oliveira GA, Oliveira CCV, Rey S, MacKenzie S, Oliveira R. Cognitive appraisal in fish: stressor predictability modulates the physiological and neurobehavioural stress response in sea bass. *Proceedings of the Royal Society B: Biological Sciences* 287, 20192922, doi:10.1098/rspb.2019.2922, 2020

Chakravarty S, Reddy BR, Sudhakar SR, Saxena S, Das T, Meghah V, Brahmendra Swamy CV, Kumar A, Idris MM. Chronic unpredictable stress (CUS)-induced anxiety and related mood disorders in a zebrafish model: altered brain proteome profile implicates mitochondrial dysfunction. *PLOS ONE* 8, e63302, doi:10.1371/journal.pone.0063302, 2013

Chatelain M, Drobniak SM, Szulkin M. The association between stressors and telomeres in non-human vertebrates: a meta-analysis. *Ecology Letters* 23, 381-98, doi:10.1111/ele.13426, 2020

Chawla R, Azzalin CM. The telomeric transcriptome and SMG proteins at the crossroads. *Cytogenetic and genome research* 122, 194-201, 2008

Childs BG, Durik M, Baker DJ, van Deursen JM. Cellular senescence in aging and age-related disease: from mechanisms to therapy. *Nature Medicine* 21, 1424, doi:10.1038/nm.4000, 2015

Choi J, Fauce SR, Effros RB. Reduced telomerase activity in human T lymphocytes exposed to cortisol. *Brain, Behavior, and Immunity* 22, 600-5, doi:https://doi.org/10.1016/j.bbi.2007.12.004, 2008

Cifuentes-Rojas C, Shippen DE. Telomerase regulation. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 730, 20-7, doi:https://doi.org/10.1016/j.mrfmmm.2011.10.003, 2012

Costantini D, Marasco V, Møller AP. A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* 181, 447-56, doi:10.1007/s00360-011-0566-2, 2011

Dai YJ, Jia YF, Chen N, Bian WP, Li QK, Ma YB, Chen YL, Pei DS. Zebrafish as a model system to study toxicology. *Environmental Toxicology and Chemistry* 33, 11-7, doi:doi:10.1002/etc.2406, 2014

Dalla C, Edgecomb C, Whetstone AS, Shors TJ. Females do not express learned helplessness like males do. *Neuropsychopharmacology* 33, 1559-69, doi:10.1038/sj.npp.1301533, 2008

Dawkins MS. Behaviour as a tool in the assessment of animal welfare. *Zoology* 106, 383-7, doi:https://doi.org/10.1078/0944-2006-00122, 2003

de Abechuco EL, Hartmann N, Soto M, Díez G. Assessing the variability of telomere length measures by means of Telomeric Restriction Fragments (TRF) in different tissues of cod *Gadus morhua*. *Gene Reports* 5, 117-25, doi:https://doi.org/10.1016/j.genrep.2016.09.009, 2016

de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes & Development* 19, 2100-10, 2005

de Lange T. Shelterin-Mediated Telomere Protection. *Annual Review of Genetics* 52, 223-47, doi:10.1146/annurev-genet-032918-021921, 2018

de Punder K, Heim C, Wadhwa PD, Entringer S. Stress and immunosenescence: The role of telomerase. *Psychoneuroendocrinology* 101, 87-100, doi:10.1016/j.psyneuen.2018.10.019, 2019

de Vere A, J., Kuczaj S, A. Where are we in the study of animal emotions? *Wiley Interdisciplinary Reviews: Cognitive Science* 7, 354-62, doi:10.1002/wcs.1399, 2016

Dedovic K, Duchesne A, Andrews J, Engert V, Pruessner JC. The brain and the stress axis: The neural correlates of cortisol regulation in response to stress. *NeuroImage* 47, 864-71, doi:https://doi.org/10.1016/j.neuroimage.2009.05.074, 2009

Dhabhar FS. Acute stress enhances while chronic stress suppresses skin immunity: the role of stress hormones and leukocyte trafficking. *Annals of the New York Academy of Sciences* 917, 876-93, doi:10.1111/j.1749-6632.2000.tb05454.x, 2006

Dickerson SS, Kemeny ME. Acute stressors and cortisol responses: a theoretical integration and synthesis of laboratory research. *Psychological Bulletin* 130, 355, 2004

Duncan IJH. The interpretation of preference tests in animal behaviour. *Applied Animal Ethology* 4, 197-200, 1978

Ellis T, Yildiz HY, López-Olmeda J, Spedicato MT, Tort L, Øverli Ø, Martins CIM. Cortisol and finfish welfare. *Fish Physiology and Biochemistry* 38, 163-88, doi:10.1007/s10695-011-9568-y, 2012

Elwood RW. Pain and suffering in invertebrates? *ILAR Journal* 52, 175-84, 2011

Epel ES, Lin J, Dhabhar FS, Wolkowitz OM, Puterman E, Karan L, Blackburn EH. Dynamics of telomerase activity in response to acute psychological stress. *Brain, Behavior, and Immunity* 24, 531-9, doi:https://doi.org/10.1016/j.bbi.2009.11.018, 2010

Forkman B, Boissy A, Meunier-Salaün MC, Canali E, Jones RB. A critical review of fear tests used on cattle, pigs, sheep, poultry and horses. *Physiology & Behavior* 92, 340-74, doi:10.1016/j.physbeh.2007.03.016, 2007

Fraser D, Weary DM, Pajor EA, Milligan BN. A scientific conception of animal welfare that reflects ethical concerns. *Animal Welfare* 6, 187-205, 1997

Froehlich JM, Fowler ZG, Galt NJ, Smith Jr DL, Biga PR. Sarcopenia and piscines: the case for indeterminate-growing fish as unique genetic model organisms in aging and longevity research. *Frontiers in Genetics* 4, 159, doi:10.3389/fgene.2013.00159, 2013

Fulcher N, Tran S, Shams S, Chatterjee D, Gerlai R. Neurochemical and behavioral responses to unpredictable chronic mild stress following developmental isolation: the zebrafish as a model for major depression. *Zebrafish* 14, 23-34, 2017

Galhardo L, Oliveira RF. Psychological stress and welfare in fish. *Annual Review of Biomedical Sciences* 11, 1-20, 2009

Galhardo L, Vital J, Oliveira RF. The role of predictability in the stress response of a cichlid fish. *Physiology & Behavior* 102, 367-72, 2011

Gesto M, Hernández J, López-Patiño MA, Soengas JL, Míguez JM. Is gill cortisol concentration a good acute stress indicator in fish? A study in rainbow trout and zebrafish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 188, 65-9, doi:https://doi.org/10.1016/j.cbpa.2015.06.020, 2015

Gilmour KM. Mineralocorticoid receptors and hormones: fishing for answers. *Endocrinology* 146, 44-6, doi:10.1210/en.2004-1390, 2005

Gopalakrishnan S, Cheung NKM, Yip BWP, Au DWT. Medaka fish exhibits longevity gender gap, a natural drop in estrogen and telomere shortening during aging: a unique model for studying sex-dependent longevity. *Frontiers in Zoology* 10, 78, doi:10.1186/1742-9994-10-78, 2013

Gorissen M, Flik G. 3 - The endocrinology of the stress response in fish: An adaptation-physiological view. In: Schreck CB, Tort L, Farrell AP, Brauner CJ (eds). *Fish Physiology*. Pp 75-111. Academic Press, 2016

Graham C, von Keyserlingk MAG, Franks B. Zebrafish welfare: Natural history, social motivation and behaviour. *Applied Animal Behaviour Science* 200, 13-22, doi:10.1016/j.applanim.2017.11.005, 2018a

Graham C, von Keyserlingk MAG, Franks B. Free-choice exploration increases affiliative behaviour in zebrafish. *Applied Animal Behaviour Science*, doi:https://doi.org/10.1016/j.applanim.2018.02.005, 2018b

Gronquist D, Berges JA. Effects of aquarium-related stressors on the zebrafish: a comparison of behavioral, physiological, and biochemical indicators. *Journal of Aquatic Animal Health* 25, 53-65, doi:10.1080/08997659.2012.747450, 2013

Gutierrez-Rodrigues F, Santana-Lemos BA, Scheucher PS, Alves-Paiva RM, Calado RT. Direct comparison of flow-FISH and qPCR as diagnostic tests for telomere length measurement in humans. *PLOS ONE* 9, 2014

Hall ME, Nasir L, Daunt F, Gault EA, Croxall JP, Wanless S, Monaghan P. Telomere loss in relation to age and early environment in long-lived birds. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 271, 1571-6, doi:10.1098/rspb.2004.2768, 2004

Harris AP, D'Eath RB, Healy SD. Environmental enrichment enhances spatial cognition in rats by reducing thigmotaxis (wall hugging) during testing. *Animal Behaviour* 77, 1459-64, 2009

Harris J, Bird DJ. Alpha-melanocyte stimulating hormone (α-MSH) and melanin-concentrating hormone (MCH) stimulate phagocytosis by head kidney leucocytes of rainbow trout (*Oncorhynchus mykiss*) in vitro. *Fish & Shellfish Immunology* 8, 631-8, doi:https://doi.org/10.1006/fsim.1998.0172, 1998

Hartmann N, Reichwald K, Lechel A, Graf M, Kirschner J, Dorn A, Terzibasi E, Wellner J, Platzer M, Rudolph KL, Cellerino A, Englert C. Telomeres shorten while Tert expression increases during ageing of the short-lived fish *Nothobranchius furzeri*. *Mechanisms of Ageing and Development* 130, 290-6, doi:https://doi.org/10.1016/j.mad.2009.01.003, 2009

Harvey MA, Beausoleil JN, Ramp D, Mellor JD. A ten-stage protocol for assessing the welfare of individual non-captive wild animals: free-roaming horses (*Equus ferus caballus*) as an example. *Animals* 10, doi:10.3390/ani10010148, 2020

Hatakeyama H, Nakamura K-I, Izumiyama-Shimomura N, Ishii A, Tsuchida S, Takubo K, Ishikawa N. The teleost *Oryzias latipes* shows telomere shortening with age despite considerable telomerase activity throughout life. *Mechanisms of Ageing and Development* 129, 550-7, doi:https://doi.org/10.1016/j.mad.2008.05.006, 2008

Hatakeyama H, Yamazaki H, Nakamura K-I, Izumiyama-Shimomura N, Aida J, Suzuki H, Tsuchida S, Matsuura M, Takubo K, Ishikawa N. Telomere attrition and restoration in the normal teleost *Oryzias latipes* are linked to growth rate and telomerase activity at each life stage. *Aging* 8, 62-75, doi:10.18632/aging.100873, 2016

Hathcock KS, Jeffrey Chiang Y, Hodes RJ. In vivo regulation of telomerase activity and telomere length. *Immunological Reviews* 205, 104-13, doi:10.1111/j.0105-2896.2005.00267.x, 2005

Haussmann MF, Marchetto NM. Telomeres: Linking stress and survival, ecology and evolution. *Current Zoology* 56, 2010

Henriques CM, Carneiro MC, Tenente IM, Jacinto A, Ferreira MG. Telomerase is required for zebrafish lifespan. *PLOS Genetics* 9, e1003214, doi:10.1371/journal.pgen.1003214, 2013

Herman JP, McKlveen JM, Ghosal S, Kopp B, Wulsin A, Makinson R, Scheimann J, Myers B. Regulation of the hypothalamic-pituitary-adrenocortical stress response. *Comprehensive Physiology* 6, 603-21, doi:10.1002/cphy.c150015, 2016

Home Office. *Annual Statistics of Scientific Procedures on Living Animals: Great Britain 2018.* Stationery Office, 2018

Horn T, Gemmell NJ, Robertson BC, Bridges CR. Telomere length change in European sea bass (*Dicentrarchus labrax*). *Australian Journal of Zoology* 56, 207-10, 2008

Igarashi H, Sakaguchi N. Telomerase activity is induced in human peripheral B lymphocytes by the stimulation to antigen receptor. *Blood* 89, 1299-307, doi:10.1182/blood.V89.4.1299, 1997

Ilmonen P, Kotrschal A, Penn DJ. Telomere attrition due to infection. *PLOS ONE* 3, e2143-e, doi:10.1371/journal.pone.0002143, 2008

Ingles ED, Deakin JE. Telomeres, species differences, and unusual telomeres in vertebrates: presenting challenges and opportunities to understanding telomere dynamics. *AIMS Genetics* 3, 1-24, doi:http://dx.doi.org/10.3934/genet.2016.1.1, 2016

Inoue T, Tsuchiya K, Koyama T. Regional changes in dopamine and serotonin activation with various intensity of physical and psychological stress in the rat brain. *Pharmacology Biochemistry and Behavior* 49, 911-20, doi:https://doi.org/10.1016/0091-3057(94)90243-7, 1994

Jensen MB, Pedersen LJ. Using motivation tests to assess ethological needs and preferences. *Applied Animal Behaviour Science* 113, 340-56, doi:10.1016/j.applanim.2008.02.001, 2008

Kimura M, Stone RC, Hunt SC, Skurnick J, Lu X, Cao X, Harley CB, Aviv A. Measurement of telomere length by the Southern blot analysis of terminal restriction fragment lengths. *Nature Protocols* 5, 1596-607, doi:10.1038/nprot.2010.124, 2010

Kistler C, Hegglin D, Würbel H, König B. Preference for structured environment in zebrafish (*Danio rerio*) and checker barbs (*Puntius oligolepis*). *Applied Animal Behaviour Science* 135, 318-27, doi:10.1016/j.applanim.2011.10.014, 2011

Koetsier G, Cantor E. A practical guide to analyzing nucleic acid concentration and purity with microvolume spectrophotometers. New England BioLabs. *Inc.: Ipswich, MA, USA*, 2019

Korte SM, Olivier B, Koolhaas JM. A new animal welfare concept based on allostasis. *Physiology & Behavior* 92, 422-8, doi:https://doi.org/10.1016/j.physbeh.2006.10.018, 2007

Kotrschal A, Ilmonen P, Penn DJ. Stress impacts telomere dynamics. *Biology Letters* 3, 128, 2007

Kulczykowska E. Responses of circulating arginine vasotocin, isotocin, and melatonin to osmotic and disturbance stress in rainbow trout (*Oncorhynchus mykiss*). *Fish Physiology and Biochemistry* 24, 201-6, doi:10.1023/A:1014079917644, 2001

Kurz DJ, Decary S, Hong Y, Trivier E, Akhmedov A, Erusalimsky JD. Chronic oxidative stress compromises telomere integrity and accelerates the onset of senescence in human endothelial cells. *Journal of Cell Science* 117, 2417, doi:10.1242/jcs.01097, 2004

Ladewig J. Chronic intermittent stress: a model for the study of long-term stressors. *The biology of animal stress*, 159-69, 2000

Lai T-P, Wright WE, Shay JW. Comparison of telomere length measurement methods. *Philosophical Transactions of the Royal Society B: Biological Sciences* 373, 20160451, doi:10.1098/rstb.2016.0451, 2018

Lawrence C. The husbandry of zebrafish (*Danio rerio*): A review. *Aquaculture* 269, 1-20, doi:https://doi.org/10.1016/j.aquaculture.2007.04.077, 2007

Lawrence C, Mason T. Zebrafish housing systems: A review of basic operating principles and considerations for design and functionality. *ILAR Journal* 53, 179-91, doi:10.1093/ilar.53.2.179, 2012

Lawrence MJ, Jain-Schlaepfer S, Zolderdo AJ, Algera DA, Gilmour KM, Gallagher AJ, Cooke SJ. Are 3 minutes good enough for obtaining baseline physiological samples from teleost fish? *Canadian Journal of Zoology* 96, 774-86, 2018

Lazzerini-Denchi E, Sfeir A. Stop pulling my strings — what telomeres taught us about the DNA damage response. *Nature Reviews Molecular Cell Biology* 17, 364-78, doi:10.1038/nrm.2016.43, 2016

Lee DY, Kim E, Choi MH. Technical and clinical aspects of cortisol as a biochemical marker of chronic stress. *BMB reports* 48, 209-16, doi:10.5483/bmbrep.2015.48.4.275, 2015

Lee H-W, Blasco MA, Gottlieb GJ, Horner JW, Greider CW, DePinho RA. Essential role of mouse telomerase in highly proliferative organs. *Nature* 392, 569-74, doi:10.1038/33345, 1998

Lidster K, Readman GD, Prescott MJ, Owen SF. International survey on the use and welfare of zebrafish *Danio rerio* in research. *Journal of Fish Biology* 90, 1891-905, doi:doi:10.1111/jfb.13278, 2017

Lin J, Smith DL, Esteves K, Drury S. Telomere length measurement by qPCR–Summary of critical factors and recommendations for assay design. *Psychoneuroendocrinology* 99, 271-8, 2019

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* 25, 402-8, doi:https://doi.org/10.1006/meth.2001.1262, 2001

LoCoco PM, Boyd JT, Espitia Olaya CM, Furr AR, Garcia DK, Weldon KS, Zou Y, Locke E, Tobon A, Lai Z, Ruparel SB, Ruparel NB, Hargreaves KM. Reliable approaches to extract high-integrity RNA from skin and other pertinent tissues used in pain research. *PAIN Reports* 5, 2020

López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The Hallmarks of Aging. *Cell* 153, 1194-217, doi:https://doi.org/10.1016/j.cell.2013.05.039, 2013

Lund TC, Glass TJ, Tolar J, Blazar BR. expression of telomerase and telomere length are unaffected by either age or limb regeneration in *Danio rerio*. *PLOS ONE* 4, e7688, doi:10.1371/journal.pone.0007688, 2009

Madaro A, Olsen RE, Kristiansen TS, Ebbesson LOE, Nilsen TO, Flik G, Gorissen M. Stress in Atlantic salmon: response to unpredictable chronic stress. *The Journal of Experimental Biology* 218, 2538, 2015

Maestripieri D, Hoffman CL. Chronic stress, allostatic load, and aging in nonhuman primates. *Development and psychopathology* 23, 1187-95, doi:10.1017/S0954579411000551, 2011

Makowska IJ, Weary DM. The importance of burrowing, climbing and standing upright for laboratory rats. *Royal Society Open Science* 3, 160136, doi:10.1098/rsos.160136, 2016

Manuel R, Gorissen M, Zethof J, Ebbesson LOE, van de Vis H, Flik G, van den Bos R. Unpredictable chronic stress decreases inhibitory avoidance learning in Tuebingen Long-Fin zebrafish (*Danio rerio* Hamilton): stronger effects in the resting phase than in the active phase. *The Journal of Experimental Biology*, 2014

Marcon M, Herrmann AP, Mocelin R, Rambo CL, Koakoski G, Abreu MS, Conterato GMM, Kist LW, Bogo MR, Zanatta L, Barcellos LJG, Piato AL. Prevention of unpredictable chronic stress-related phenomena in

zebrafish exposed to bromazepam, fluoxetine and nortriptyline. *Psychopharmacology* 233, 3815-24, doi:10.1007/s00213-016-4408-5, 2016

Marcon M, Mocelin R, Benvenutti R, Costa T, Herrmann AP, de Oliveira DL, Koakoski G, Barcellos LJG, Piato A. Environmental enrichment modulates the response to chronic stress in zebrafish. *The Journal of Experimental Biology* 221, 2018a

Marcon M, Mocelin R, Sachett A, Siebel AM, Herrmann AP, Piato A. Enriched environment prevents oxidative stress in zebrafish submitted to unpredictable chronic stress. *PeerJ* 6, e5136, doi:10.7717/peerj.5136, 2018b

Matthews M, Trevarrow B, Matthews J. A virtual tour of the guide for zebrafish users. *Lab Animal* 31, 34-40, 2002

McChesney PA, Elmore LW, Holt SE. Vertebrate marine species as model systems for studying telomeres and telomerase. *Zebrafish* 1, 349-55, doi:10.1089/zeb.2005.1.349, 2005

McCormick SD. Endocrine control of osmoregulation in teleost fish. *American Zoologist* 41, 781-94, doi:10.1093/icb/41.4.781, 2001

McCurley AT, Callard GV. Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. *BMC Molecular Biology* 9, 102, doi:10.1186/1471-2199-9-102, 2008

Mellor D, Reid C. Concepts of animal well-being and predicting the impact of procedures on experimental animals. *Improving the well-being of animals in the research environment*, 3-18, 1994

Mellor DJ, Cook CJ, Stafford KJ. Quantifying some responses to pain as a stressor. *The Biology of Animal Stress: Basic Principles and Implications for Welfare*, 171-98, 2000

Mellor DJ, Patterson-Kane E, Stafford KJ. *The sciences of animal welfare.* Oxford ; Ames, Iowa : Wiley-Blackwell, 2009

Mellor DJ. Enhancing animal welfare by creating opportunities for positive affective engagement. *NZ Vet J* 63, 3-8, doi:10.1080/00480169.2014.926799, 2015

Mellor DJ, Beausoleil NJ. Extending the 'Five Domains' model for animal welfare assessment to incorporate positive welfare states. *Animal Welfare* 24, 241-53, doi:10.7120/09627286.24.3.241, 2015

Mellor DJ. Updating animal welfare thinking: moving beyond the "five freedoms" towards "a life worth living". *Animals* 6, doi:10.3390/ani6030021, 2016

Mellor DJ. Operational details of the five domains model and its key applications to the assessment and management of animal welfare. *Animals (Basel)* 7, doi:10.3390/ani7080060, 2017

Mender I, Shay JW. Telomere restriction fragment (TRF) analysis. *Bio-protocol* 5, e1658, doi:10.21769/bioprotoc.1658, 2015

Meshalkina DA, Kizlyk MN, Kysil EV, Collier AD, Echevarria DJ, Abreu MS, Barcellos LJG, Song C, Kalueff AV. Understanding zebrafish cognition. *Behavioural Processes* 141, 229-41, doi:https://doi.org/10.1016/j.beproc.2016.11.020, 2017

Metz JR, van den Burg EH, Bonga SEW, Flik G. Regulation of branchial Na+K+-ATPase in common carp (*Cyprinus carpio*) acclimated to different temperatures. *Journal of Experimental Biology* 206, 2273, 2003

Moberg GP, Mench JA. *The biology of animal stress: basic principles and implications for animal welfare.* CABI, 2000

Molinero A, Gomez E, Balasch J, Tort L. Stress by fish removal in the gilthead sea bream, *Sparus aurata*. *Journal of Applied Aquaculture* 7, 1-12, doi:10.1300/J028v07n02_01, 1997

Monaghan P. Organismal stress, telomeres and life histories. *The Journal of Experimental Biology* 217, 57, 2014

Montpetit AJ, Alhareeri AA, Montpetit M, Starkweather AR, Elmore LW, Filler K, Mohanraj L, Burton CW, Menzies VS, Lyon DE, Collins JB, Teefey JM, Jackson-Cook CK. Telomere length: a review of methods for measurement. *Nursing research* 63, 289-99, doi:10.1097/NNR.0000000000000037, 2014

Mundstock E, Zatti H, Louzada FM, Oliveira SG, Guma FTCR, Paris MM, Rueda AB, Machado DG, Stein RT, Jones MH, Sarria EE, Barbé-Tuana FM, Mattiello R. Effects of physical activity in telomere length: Systematic review and meta-analysis. *Ageing Research Reviews* 22, 72-80, doi:https://doi.org/10.1016/j.arr.2015.02.004, 2015

Nardocci G, Navarro C, Cortés PP, Imarai M, Montoya M, Valenzuela B, Jara P, Acuña-Castillo C, Fernández R. Neuroendocrine mechanisms for immune system regulation during stress in fish. *Fish & Shellfish Immunology* 40, 531-8, doi:https://doi.org/10.1016/j.fsi.2014.08.001, 2014

Nasiadka A, Clark MD. Zebrafish breeding in the laboratory environment. *ILAR Journal* 53, 161-8, doi:10.1093/ilar.53.2.161, 2012

Nettle D, Monaghan P, Gillespie R, Brilot B, Bedford T, Bateson M. An experimental demonstration that early-life competitive disadvantage accelerates telomere loss. *Proceedings of the Royal Society B: Biological Sciences* 282, 2015

Nettle D, Andrews C, Reichert S, Bedford T, Kolenda C, Parker C, Martin-Ruiz C, Monaghan P, Bateson M. Early-life adversity accelerates cellular ageing and affects adult inflammation: Experimental evidence from the European starling. *Scientific Reports* 7, 40794, doi:10.1038/srep40794 https://www.nature.com/articles/srep40794#supplementary-information, 2017

Nicol CJ, Caplen G, Edgar J, Browne WJ. Associations between welfare indicators and environmental choice in laying hens. *Animal Behaviour* 78, 413-24, doi:10.1016/j.anbehav.20, 2009

O'Callaghan NJ, Fenech M. A quantitative PCR method for measuring absolute telomere length. *Biological Procedures Online* 13, 3, doi:10.1186/1480-9222-13-3, 2011

Oka S, Chapman CR, Kim B, Shimizu O, Noma N, Takeichi O, Imamura Y, Oi Y. Predictability of painful stimulation modulates subjective and physiological responses. *The Journal of Pain* 11, 239-46, doi:https://doi.org/10.1016/j.jpain.2009.07.009, 2010

Olsson M, Pauliny A, Wapstra E, Blomqvist D. Proximate determinants of telomere length in sand lizards (*Lacerta agilis*). *Biology Letters* 6, 651-3, doi:10.1098/rsbl.2010.0126, 2010

Olsson M, Pauliny A, Wapstra E, Uller T, Schwartz T, Miller E, Blomqvist D. Sexual differences in telomere selection in the wild. *Molecular Ecology* 20, 2085-99, doi:10.1111/j.1365-294X.2011.05085.x, 2011

Olsson M, Wapstra E, Friesen C. Ectothermic telomeres: it's time they came in from the cold. *Philosophical Transactions of the Royal Society B: Biological Sciences* 373, 2018

Parichy DM, Elizondo MR, Mills MG, Gordon TN, Engeszer RE. Normal table of postembryonic zebrafish development: staging by externally visible anatomy of the living fish. *Developmental dynamics : an official publication of the American Association of Anatomists* 238, 2975-3015, doi:10.1002/dvdy.22113, 2009

Pauliny A, Devlin RH, Johnsson JI, Blomqvist D. Rapid growth accelerates telomere attrition in a transgenic fish. *BMC Evolutionary Biology* 15, 159, doi:10.1186/s12862-015-0436-8, 2015

Pavlidis M, Theodoridi A, Tsalafouta A. Neuroendocrine regulation of the stress response in adult zebrafish, *Danio rerio*. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 60, 121-31, doi:https://doi.org/10.1016/j.pnpbp.2015.02.014, 2015

Peterson DR, Mok HOL, Au DWT. Modulation of telomerase activity in fish muscle by biological and environmental factors. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 178, 51-9, doi:https://doi.org/10.1016/j.cbpc.2015.09.004, 2015

Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research* 29, e45-e, doi:10.1093/nar/29.9.e45, 2001

Piato ÂL, Capiotti KM, Tamborski AR, Oses JP, Barcellos LJG, Bogo MR, Lara DR, Vianna MR, Bonan CD. Unpredictable chronic stress model in zebrafish (*Danio rerio*): Behavioral and physiological responses. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 35, 561-7, doi:https://doi.org/10.1016/j.pnpbp.2010.12.018, 2011

Pickering AD, Pottinger TG. Stress responses and disease resistance in salmonid fish: Effects of chronic elevation of plasma cortisol. *Fish Physiology and Biochemistry* 7, 253-8, doi:10.1007/BF00004714, 1989

Portavella M, Torres B, Salas C. Avoidance response in goldfish: emotional and temporal involvement of medial and lateral telencephalic pallium. *The Journal of Neuroscience* 24, 2335, doi:10.1523/JNEUROSCI.4930-03.2004, 2004

Prunet P, Sturm A, Milla S. Multiple corticosteroid receptors in fish: From old ideas to new concepts. *General and Comparative Endocrinology* 147, 17-23, doi:https://doi.org/10.1016/j.ygcen.2006.01.015, 2006

Rambo CL, Mocelin R, Marcon M, Villanova D, Koakoski G, de Abreu MS, Oliveira TA, Barcellos LJG, Piato AL, Bonan CD. Gender differences in aggression and cortisol levels in zebrafish subjected to unpredictable chronic stress. *Physiology & Behavior* 171, 50-4, doi:https://doi.org/10.1016/j.physbeh.2016.12.032, 2017

Ramsay JM, Feist GW, Varga ZM, Westerfield M, Kent ML, Schreck CB. Whole-body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. *Aquaculture* 258, 565-74, doi:https://doi.org/10.1016/j.aquaculture.2006.04.020, 2006

Ramsay JM, Feist GW, Varga ZM, Westerfield M, Kent ML, Schreck CB. Whole-body cortisol response of zebrafish to acute net handling stress. *Aquaculture* 297, 157-62, doi:https://doi.org/10.1016/j.aquaculture.2009.08.035, 2009

Raschenberger J, Lamina C, Haun M, Kollerits B, Coassin S, Boes E, Kedenko L, Köttgen A, Kronenberg F. Influence of DNA extraction methods on relative telomere length measurements and its impact on epidemiological studies. *Scientific Reports* 6, 25398, doi:10.1038/srep25398, 2016

Reed B, Jennings M. *Guidance on the housing and care of zebrafish Danio rerio.* Royal Society for the Prevention of Cruelty to Animals (RSPCA), 2011

Reid SG, Bernier NJ, Perry SF. The adrenergic stress response in fish: control of catecholamine storage and release. Communicated by Dr P.W. Hochachka, Editor.1. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* 120, 1-27, doi:https://doi.org/10.1016/S0742-8413(98)00037-1, 1998

Ribas L, Piferrer F. The zebrafish (*Danio rerio*) as a model organism, with emphasis on applications for finfish aquaculture research. *Reviews in Aquaculture* 6, 209-40, doi:10.1111/raq.12041, 2014

Sadoul B, Geffroy B. Measuring cortisol, the major stress hormone in fishes. *Journal of Fish Biology* 94, 540-55, doi:10.1111/jfb.13904, 2019

Salomons HM, Mulder GA, van de Zande L, Haussmann MF, Linskens MHK, Verhulst S. Telomere shortening and survival in free-living corvids. *Proceedings of the Royal Society B: Biological Sciences* 276, 3157-65, doi:10.1098/rspb.2009.0517, 2009

Samaras A, Espirito Santo C, Papandroulakis N, Mitrizakis N, Pavlidis M, Höglund E, Pelgrim TNM, Zethof J, Spanings TFA, Vindas MA. Allostatic load and stress physiology in European seabass (*Dicentrarchus labrax* L.) and Gilthead seabream (*Sparus aurata* L.). *Frontiers in Endocrinology* 9, 451, 2018

Santos GA, Schrama JW, Mamauag REP, Rombout JHWM, Verreth JAJ. Chronic stress impairs performance, energy metabolism and welfare indicators in European seabass (*Dicentrarchus labrax*): The combined effects of fish crowding and water quality deterioration. *Aquaculture* 299, 73-80, doi:https://doi.org/10.1016/j.aquaculture.2009.11.018, 2010

Schreck CB. Accumulation and long-term effects of stress in fish. *The biology of animal stress* 1, 147-58, 2000

Schreck CB, Tort L. 1 - The Concept of stress in fish. In: Schreck CB, Tort L, Farrell AP, Brauner CJ (eds). *Fish Physiology*. Pp 1-34. Academic Press, 2016

Schroeder P, Jones S, Young IS, Sneddon LU. What do zebrafish want? Impact of social grouping, dominance and gender on preference for enrichment. *Laboratory Animals* 48, 328-37, doi:10.1177/0023677214538239, 2014

Seferta A, Guay PJ, Marzinotto E, Lefebvre L. Learning differences between feral pigeons and Zenaida doves: the role of neophobia and human proximity. *Ethology* 107, 281-93, doi:10.1046/j.1439-0310.2001.00658.x, 2001

Shay JW, Wright WE. Hayflick, his limit, and cellular ageing. *Nature Reviews Molecular Cell Biology* 1, 72, doi:10.1038/35036093, 2000

Shay JW, Wright WE. Telomeres and telomerase: three decades of progress. *Nature Reviews Genetics* 20, 299-309, doi:10.1038/s41576-019-0099-1, 2019

Sikora E, Arendt T, Bennett M, Narita M. Impact of cellular senescence signature on ageing research. *Ageing Research Reviews* 10, 146-52, doi:https://doi.org/10.1016/j.arr.2010.10.002, 2011

Simide R, Angelier F, Gaillard S, Stier A. Age and heat stress as determinants of telomere length in a long-lived fish, the siberian sturgeon. *Physiological and Biochemical Zoology* 89, 441-7, doi:10.1086/687378, 2016

Sink TD, Kumaran S, Lochmann RT. Development of a whole-body cortisol extraction procedure for determination of stress in golden shiners,

Notemigonus crysoleucas. *Fish Physiology and Biochemistry* 33, 189-93, doi:10.1007/s10695-007-9130-0, 2007

Smith SM, Vale WW. The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. *Dialogues in clinical neuroscience* 8, 383-95, 2006

Sneddon LU, Wolfenden DCC, Leach MC, Valentim AM, Steenbergen PJ, Bardine N, Broom DM, Brown C. Ample evidence for fish sentience and pain. *Animal Sentience* 3, 17, 2018

Sohn SH, Subramani VK, Moon YS, Jang IS. Telomeric DNA quantity, DNA damage, and heat shock protein gene expression as physiological stress markers in chickens. *Poultry Science* 91, 829-36, doi:https://doi.org/10.3382/ps.2011-01904, 2012

Song C, Liu B-P, Zhang Y-P, Peng Z, Wang J, Collier AD, Echevarria DJ, Savelieva KV, Lawrence RF, Rex CS, Meshalkina DA, Kalueff AV. Modeling consequences of prolonged strong unpredictable stress in zebrafish: Complex effects on behavior and physiology. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 81, 384-94, doi:https://doi.org/10.1016/j.pnpbp.2017.08.021, 2018

Sopinka NM, Donaldson MR, O'Connor CM, Suski CD, Cooke SJ. 11 - Stress indicators in fish. In: Schreck CB, Tort L, Farrell AP, Brauner CJ (eds). *Fish Physiology*. Pp 405-62. Academic Press, 2016

Speedie N, Gerlai R. Alarm substance induced behavioral responses in zebrafish (*Danio rerio*). *Behavioural Brain Research* 188, 168-77, doi:https://doi.org/10.1016/j.bbr.2007.10.031, 2008

Spence R, Smith C. Male territoriality mediates density and sex ratio effects on oviposition in the zebrafish, *Danio rerio*. *Animal Behaviour* 69, 1317-23, doi:https://doi.org/10.1016/j.anbehav.2004.10.010, 2005

Stam R, Bruijnzeel AW, Wiegant VM. Long-lasting stress sensitisation. *European Journal of Pharmacology* 405, 217-24, doi:https://doi.org/10.1016/S0014-2999(00)00555-0, 2000

Steenbergen PJ, Richardson MK, Champagne DL. The use of the zebrafish model in stress research. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 35, 1432-51, doi:https://doi.org/10.1016/j.pnpbp.2010.10.010, 2011

Stindl R. Tying it all together: telomeres, sexual size dimorphism and the gender gap in life expectancy. *Medical Hypotheses* 62, 151-4, doi:https://doi.org/10.1016/S0306-9877(03)00316-5, 2004

Stolte EH, de Mazon AF, Leon-Koosterziel KM, Jesiak M, Bury NR, Sturm A, Savelkoul HFJ, van Kemenade BMLV, Flik G. Corticosteroid receptors involved in stress regulation in common carp, *Cyprinus carpio*. *The Journal of endocrinology* 198, 403-17, doi:10.1677/joe-08-0100, 2008

Suriyampola PS, Shelton DS, Shukla R, Roy T, Bhat A, Martins EP. Zebrafish social behavior in the wild. *Zebrafish* 13, 1-8, doi:10.1089/zeb.2015.1159, 2015

Svec D, Tichopad A, Novosadova V, Pfaffl MW, Kubista M. How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. *Biomolecular detection and quantification* 3, 9-16, doi:10.1016/j.bdq.2015.01.005, 2015

Takahashi A, Kobayashi Y, Mizusawa K. The pituitary–interrenal axis of fish: A review focusing on the lamprey and flounder. *General and Comparative*

Endocrinology 188, 54-9, doi:https://doi.org/10.1016/j.ygcen.2013.03.005, 2013

Toni M, Manciocco A, Angiulli E, Alleva E, Cioni C, Malavasi S. Review: Assessing fish welfare in research and aquaculture, with a focus on European directives. *animal* 13, 161-70, doi:10.1017/S1751731118000940, 2019

Tran S, Gerlai R. Individual differences in activity levels in zebrafish (*Danio rerio*). *Behavioural Brain Research* 257, 224-9, doi:https://doi.org/10.1016/j.bbr.2013.09.040, 2013

Turnbull J, Bell A, Adams C, Bron J, Huntingford F. Stocking density and welfare of cage farmed Atlantic salmon: application of a multivariate analysis. *Aquaculture* 243, 121-32, doi:https://doi.org/10.1016/j.aquaculture.2004.09.022, 2005

Ujvari B, Madsen T. Short telomeres in hatchling snakes: erythrocyte telomere dynamics and longevity in tropical pythons. *PLOS ONE* 4, e7493-e, doi:10.1371/journal.pone.0007493, 2009

van de Nieuwegiessen PG, Boerlage AS, Verreth JAJ, Schrama JW. Assessing the effects of a chronic stressor, stocking density, on welfare indicators of juvenile African catfish, *Clarias gariepinus* Burchell. *Applied Animal Behaviour Science* 115, 233-43, doi:https://doi.org/10.1016/j.applanim.2008.05.008, 2008

Van Houcke J, De Groef L, Dekeyster E, Moons L. The zebrafish as a gerontology model in nervous system aging, disease, and repair. *Ageing Research Reviews* 24, 358-68, doi:https://doi.org/10.1016/j.arr.2015.10.004, 2015

Vansteenwegen D, Iberico C, Vervliet B, Marescau V, Hermans D. Contextual fear induced by unpredictability in a human fear conditioning preparation is related to the chronic expectation of a threatening US. *Biological Psychology* 77, 39-46, doi:https://doi.org/10.1016/j.biopsycho.2007.08.012, 2008

von Krogh K, Sorensen C, Nilsson GE, Overli O. Forebrain cell proliferation, behavior, and physiology of zebrafish, *Danio rerio*, kept in enriched or barren environments. *Physiology & Behaviour* 101, 32-9, doi:10.1016/j.physbeh.2010.04.003, 2010

von Zglinicki T. Oxidative stress shortens telomeres. *Trends in Biochemical Sciences* 27, 339-44, doi:https://doi.org/10.1016/S0968-0004(02)02110-2, 2002

Wagner K-D, Ying Y, Leong W, Jiang J, Hu X, Chen Y, Michiels J-F, Lu Y, Gilson E, Wagner N, Ye J. The differential spatiotemporal expression pattern of shelterin genes throughout lifespan. *Aging* 9, 1219-28, doi:10.18632/aging.101223, 2017

Webster J. Animal welfare: Freedoms, dominions and "a life worth living". *Animals* 6, doi:10.3390/ani6060035, 2016

Wellinger RJ. In the end, what's the problem? *Molecular Cell* 53, 855-6, doi:https://doi.org/10.1016/j.molcel.2014.03.008, 2014

Wendelaar Bonga SE. The stress response in fish. *Physiological Reviews* 77, 591-625, doi:10.1152/physrev.1997.77.3.591, 1997

Wilkes L, Owen SF, Readman GD, Sloman KA, Wilson RW. Does structural enrichment for toxicology studies improve zebrafish welfare? *Applied Animal Behaviour Science* 139, 143-50, doi:10.1016/j.applanim.2012.03.011, 2012

Williams TD, Readman GD, Owen SF. Key issues concerning environmental enrichment for laboratory-held fish species. *Laboratory Animals* 43, 107-20, doi:10.1258/la.2007.007023, 2009

Wilson JM, Bunte RM, Carty AJ. Evaluation of rapid cooling and tricaine methanesulfonate (MS222) as methods of euthanasia in zebrafish (*Danio rerio*). *Journal of the American Association for Laboratory Animal Science* 48, 785-9, 2009

Winberg S, Höglund E, Øverli Ø. 2 - Variation in the neuroendocrine stress response. In: Schreck CB, Tort L, Farrell AP, Brauner CJ (eds). *Fish Physiology*. Pp 35-74. Academic Press, 2016

Wofford JC, Goodwin VL. The linkages of cognitive processes, stress propensity, affect, and strain: experimental test of a cognitive model of the stress response. *Personality and Individual Differences* 32, 1413-30, doi:https://doi.org/10.1016/S0191-8869(01)00130-1, 2002

Xie Y, Yang D, He Q, Songyang Z. Zebrafish as a model system to study the physiological function of telomeric protein TPP1. *PLOS ONE* 6, e16440, doi:10.1371/journal.pone.0016440, 2011

Xin H, Liu D, Songyang Z. The telosome/shelterin complex and its functions. *Genome Biology* 9, 232, doi:10.1186/gb-2008-9-9-232, 2008

Yada T, Tort L. 10 - Stress and disease resistance: immune system and immunoendocrine interactions. In: Schreck CB, Tort L, Farrell AP, Brauner CJ (eds). *Fish Physiology*. Pp 365-403. Academic Press, 2016

Yarahmadi P, Miandare HK, Fayaz S, Caipang CMA. Increased stocking density causes changes in expression of selected stress- and immune-related genes, humoral innate immune parameters and stress responses of rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology* 48, 43-53, doi:https://doi.org/10.1016/j.fsi.2015.11.007, 2016

Yin Z, Lam TJ, Sin YM. The effects of crowding stress on the non-specific immuneresponse in fancy carp (*Cyprinus carpio* L.). *Fish & Shellfish Immunology* 5, 519-29, doi:https://doi.org/10.1016/S1050-4648(95)80052-2, 1995

Yossa R, Sarker PK, Proulx E, Saxena V, Ekker M, Vandenberg GW. A practical approach for sexing zebrafish, *Danio rerio*. *Journal of Applied Aquaculture* 25, 148-53, doi:10.1080/10454438.2013.792170, 2013

Appendix A

Fish maintenance

Schedule and protocols

Daily schedule:

Time of day		Action	
AM	**6.00**	Light intensity begins increasing	
	6.30	Lights at 100%	
	7.00	Pellet feed	
	7.30		
	8.00		
	8.30		
	9.00		AM treatment
	9.30		
	10.00		
	10.30		
	11.00		
	11.30		
PM	**12.00**		
	12.30		
	1.00	Live feed	
	1.30		
	2.00		
	2.30		PM treatment
	3.00		
	3.30		
	4.00		
	4.30		
	5.00		
	5.30		
	6.00	Water change (25%, every 2nd day)	
	6.30		
	7.00	Pellet feed	
	7.30	Light intensity begins decreasing	
	8.00	Lights off	

Protocols:

Fish arrival and sorting

Equipment

- System tank x6
- Small net
- Scissors
- Large bowl

Preparation

1. Check water temperature before floating fish
2. Ensure tanks are correctly labelled
3. Remove tanks from system

Procedure

1. Carefully cut open transport box and remove fish bag
2. Visually inspect fish and note any deaths and injuries
3. Float fish bag in bucket for one hour
   - ⇒ Add 250ml of system water every 20 minutes
4. At the end of the hour, remove fish bag from bucket and place in stable position on work bench
   - ⇒ Use bowl to stabilise bag
5. Net a group of fish and place in unlabelled system tank
6. Separate females into a second unlabelled system tank
   - ⇒ When one tank reaches density of 20 fish, start moving male/female pairs into labelled tanks
   - ⇒ Record how many pairs each tank has as you go
7. Repeat 5-6 until all fish are removed from transport bag
8. Determine sex ratio by counting males and females
9. Place remaining fish in unlabelled tanks into labelled tanks, maintaining sex ratio in each tank
   - ⇒ 19 fish per tank
   - ⇒ Record sex ratio on note sheet
   - ⇒ If stocking density is correct, 6 fish will be left in unlabelled tanks. Place these fish into a single unlabelled tank
10. Return tanks to system

Pellet feed

Twice daily, 30 minutes after lights up and 30 minutes before lights down.

Equipment

- ZM400 pellets
- Pellet dispenser

Preparation

1. Open pellet container and check for mould
2. Check that dispenser is clean and dry

Procedure

1. Fill dispenser to marked line
2. Dispense pellets through feeding hole in lid of tank
   ⇒ When feeding, take care to avoid getting pellets on the sides of the tank, or getting the dispenser wet. These actions may lead to mould growth.
3. Feed as much as fish can eat within two minutes

Live feed

Once daily at 1300h.
Two hatcheries operating 24h apart, with 48h between set-up and harvest. Each hatchery should be reset immediately after harvest.

Equipment (per hatchery)

- Canned brine shrimp (*Artemia spp.*) cysts
- Hatchery body
- Air pump
- Bottle light
- Water dropper
- Mesh sieve
- Cup
- Cleaning brush
- Mixing rod
- Cardboard tube
- Bleach
- Bucket

Preparation

One hour to half an hour before harvesting

1. Check that most cysts have hatched
    ⇒ Cysts are brown, hatched shrimp are orange/red
2. Move light position to bottom of hatchery
3. Disconnect air tube from air pump

At harvest

4. Turn off air pump and lamp
5. Place collection cup near hatchery air tube
6. Disconnect air tube from check valve and run until most hatched shrimp have been collected
    ⇒ Turn off before floating cysts are collected
7. Place lid on collection cup and put aside
    ⇒ Brine shrimp will survive a few hours in collection cup

Reset hatchery

8. Empty rest of water into a separate cup, rinse out leftover cysts
9. Rinse hatchery thoroughly with hot tap water, scrub with brush
10. Rinse hatchery with system water
11. Reconnect air pump
12. Turn lamp on and position at centre of hatchery
13. Add 250mL water from blue bin
14. Add ½ tablespoon salt and mix
15. Add ¼ teaspoon of brine shrimp cysts
16. Replace lid of hatchery
17. Place cardboard tube over hatchery
18. Note date of set up on attached post-it

Procedure

1. Pour harvested shrimp from collection cup into sieve
2. Rinse with system water
3. Reverse rinse shrimp back into collection cup
4. Leave for five minutes to settle
5. Use water dropper to pick up brine shrimp and dispense through feeding hole
    ⇒ To avoid feeding unhatched cysts, pick up shrimp from near the surface
6. Distribute evenly between tanks until only unhatched cysts remain
7. Thoroughly clean collection cup, sieve, and water dropper and leave to air dry
8. Place any unwanted cysts or shrimp into 10% bleach solution for at least 6 hours

Health check and quarantine

Visual inspection three times daily at feeding times. If multiple fish in the same tank show the same symptoms, perform a water check.

Signs of ill health

Behavioural Changes	**External Changes**
Fish at surface	Colour change
Rapid breathing/gaping	Weight loss
Lethargy	Exophalmia/Pop-eyes
Circling, twirling	Distended abdomen
Loss of equilibrium	Skeletal deformity
Rubbing on surfaces	Masses/swellings
	Haemorrhage/redness
	Gas bubbles
	Protruding scales
	Fin erosion or lesion
	Skin ulceration

Equipment

- Breeding tank outer with lid
- Correctly labelled net
- Spare system tank

Preparation

- Using red tape, label breeding tank with home tank identifier and date
- Fill breeding tank with system water
- Remove home tank from system

Procedure

When problem is first noticed

1. Remove tank from system and net affected fish
2. Move to breeding tank and place lid
3. Replace home tank on system
4. Record time, date, home tank identifier and observed health problem on note sheet
   - ⇒ If observed problem is severe or urgent, contact Morgan after separating affected fish

After 24 hours

5. Check for improvement
6. If fish is recovered, move to spare system tank
   - ⇒ Do not return fish to experimental tank
7. If fish is not recovered, contact Morgan to arrange euthanasia
   - ⇒ Do not euthanise fish without explicit consent
8. Euthanasia procedure as described in "Sampling Protocol"

Post-mortem

In the event of fish death, remove the body from the home tank immediately.

Disposal of body

1. Remove body from tank by netting
2. Place body in Ziploc bag and seal
3. Visually inspect body for any signs of injury
   ⇒ Note the tank identifier, date, time, any identified signs of injury, and the sex of the fish if possible
4. Place sealed bag in biowaste bin, found in Lab1

After disposing of the body, perform a full water check and note all results. If any parameters are outside of range, perform a 25% water change and re-test.

Water check

Water level checked and corrected twice daily after feeding.

Room and water temperature is checked twice daily at lights up and lights down.

Other parameters are checked at minimum once weekly using water taken directly from the system sump. Follow instructions provided with test kit to perform tests and record all results on check sheet.

Parameter	**Acceptable range**
Temperature (room and water)	27-28°C
pH	7-8
Ammonia	0ppm
Nitrite	0ppm
Nitrate	0 – 10ppm
Salinity/Conductivity	200-1000µS

If any parameters test outside of acceptable range, perform 25% water change.

⇒ Record time, date, incorrect parameter, and action taken on note sheet
⇒ If possible, retest in one hour. If not possible, let Morgan know

Equipment

- Thermometer
- Conductivity meter
- Master water kit

Water change

Performed as required, when indicated by incorrect water parameters.

Equipment

- Large empty bucket
- Replacement water
- Plastic tubing

Preparation

1. Locate 10L line in bucket
2. Fill tubing with water from replacement sump

Procedure

1. Using tubing, siphon water from system sump into bucket until desired amount is removed
2. Refill system sump with water from replacement sump
3. Remove and empty bucket
4. Refill replacement sump

Replacement sump

Checked after every use, refilled when water line lower than ¾

Equipment

- Carbon-filtered water
- Trolley
- Air pump
- Air pump tubing
- Bucket x4

Preparation

1. Locate temperature equilibrated (grey) buckets filled with carbon-filtered water
2. Locate fill line in sump

Procedure

1. Fill sump using temperature equilibrated water from grey buckets
2. When all buckets are empty, refill
3. Fill buckets with carbon-filtered water from sea water lab
4. Return buckets to fish room

Labelling

All tanks, nets and other tank-specific equipment are labelled. Do not mix equipment or remove labels.

C for "control" or S for "stress treatment" plus tank number are henceforth referred to as "tank identifier".

Equipment

- Yellow electrical tape
- Green electrical tape
- Red electrical tape
- Blue electrical tape
- Permanent marker
- Scissors

Home tanks

Tape on bottom left-hand corner

- Tank identifier on green tape for "control"
- Tank identifier on yellow tape for "stress treatment"

Tape on right-hand side

- "Min" on blue tape for minimum water level during air exposure treatment
- "Max" on blue tape for maintenance water level

Nets

- Blue nets with tank identifier on green tape for "control"
- Yellow nets with tank identifier on yellow tape for "stress treatment"

Euthanasia tanks

- Tank identifier on green tape for "control"
- Tank identifier on yellow tape for "stress treatment"

Quarantine tanks

- Red tape with tank identifier, symptoms, time and date of quarantine start

Appendix B

Unpredictable Chronic Stress

Schedule and Procedures

Contents

Schedule

Week		Monday		Tuesday		Wednesday		Thursday		Friday		Saturday		Sunday	
1	AM	8.30am	Low water	10am	Cooling	11.15am	Chasing	9.45am	Crowding	11am	Air exposure	10.30am	Heating	11.30am	Chasing
	PM	2pm	Cooling	3pm	Crowding	1.45pm	Heating	3.30pm	Low water	2.15pm	Cooling	4.15pm	Chasing	1.30pm	Air exposure
2	AM	9.30am	Low water	11am	Air exposure	9.45am	Cooling	8.30am	Chasing	10.45am	Heating	9.30am	Crowding	11.15am	Low water
	PM	3pm	Heating	4pm	Chasing	1.45pm	Air exposure	2.30pm	Low water	1.30pm	Crowding	3.15pm	Cooling	2pm	Air exposure
3	AM	11.15am	Crowding	10am	Cooling	9.45am	Crowding	8.30am	Low water	10.30am	Air exposure	11am	Heating	9.30am	Chasing
	PM	2.15pm	Heating	3.45pm	Low water	1.30pm	Cooling	3pm	Heating	4pm	Chasing	2pm	Crowding	3.30pm	Air exposure
4	AM	10am	Crowding	12.15pm	Low water	9.30am	Chasing	10.30am	Cooling	11am	Air exposure	9.45am	Heating	10.45am	Crowding
	PM	4pm	Chasing	2.30pm	Heating	1.30pm	Cooling	3pm	Low water	2.15pm	Crowding	3.45pm	Air exposure	2.45pm	Heating

Procedures:

Heating:

Equipment

- Polystyrene box
- Thermometer
- White electrical tape
- Permanent marker
- Tecniplast net x3
- Timer x3
- Hot water
- Drying rack

Preparation (10 min)

1. Check that all three treatment tanks are labelled with tank identifier
2. Take treatment tanks off system and place in polystyrene box
3. Place one heater in each tank and switch on at the wall
   ⇒ Please do not change temperature settings on heaters
4. Rinse nets with system water
5. Timers set for 30min

Procedure (40 min)

1. Switch off heaters and remove from tanks
2. Record the temperature of each treatment tank
3. Remove home tanks from system
4. For each tank, net all fish together and release into treatment tank
5. When all fish are in treatment tanks, start timer
6. Place lid on polystyrene box
7. Return home tanks to system, circulation on
8. Rinse nets with system water and hang up

5 min before timer ends

9. Remove home tanks from system
10. Place nets with corresponding tanks

End of timer

11. For each tank, net all fish together
12. Release into home tank
13. Return home tanks to system

Clean-up (10 min)

1. Treatment tanks emptied and cleaned
2. Nets cleaned and hung up
3. Timers put away

Cooling:

Equipment

- Polystyrene box
- Thermometer
- White electrical tape
- Permanent marker
- Tecniplast net x3
- Timer x3
- Hot water
- Drying rack
- Trolley

Preparation (10 min)

1. Check that all three treatment tanks are labelled with tank identifier
2. Take treatment tanks off system
3. Move tanks to 23°C room (TCR2, next to fish room)
4. Rinse nets with system water
5. Timers set for 30min

Procedure (40 min)

1. Collect treatment tanks from 23°C room and place inside polystyrene box
2. Record the temperature of each treatment tank
3. Remove home tanks from system
4. For each tank, net all fish together and release into treatment tank
5. When all fish are in treatment tanks, start timer
6. Place lid on polystyrene box
7. Return home tanks to system, circulation on
8. Rinse nets with system water and hang up

5 min before timer ends

9. Remove home tanks from system
10. Place nets with corresponding tanks

End of timer

11. For each tank, net all fish together
12. Release into home tank
13. Return home tanks to system

Clean-up (10 min)

4. Treatment tanks emptied and rinsed
5. Nets cleaned and hung up
6. Timers put away

Crowding:

Equipment

- Tecniplast divider x3
- Plastic divider x3
- Timer x3
- Hot water
- Drying rack

Preparation (10 min)

1. Dividers cleaned, rinsed and laid out for easy access
2. Timers set to 50min

Procedure (1 hour)

1. Remove home tank from system
2. Insert plastic divider in right side of tank
3. Move plastic divider towards centre of tank
4. When all fish are in left side of tank, insert Tecniplast divider in centre
5. Start timer
6. Return home tank to system, circulation on
7. Repeat above for tanks 2-3
8. Clean plastic dividers and place on drying rack

5 min before timer end

9. Remove home tank from system

End of timer

10. Remove divider
11. Return home tank to system

Clean-up (5 min)

1. Dividers cleaned, dried, put away
2. Timers put away

Repeated air exposure:

Equipment

- Labelled Tecniplast net x3
- Timer x6
- Tecniplast divider

Preparation (5 min)

1. Nets rinsed with system water
2. Work space cleared
3. Timers labelled, two timers to match each tank
4. 3 timers set for 1 min
5. 3 timers set for 10min

Procedure (40 min)

1. Remove home tanks from system
2. Net all fish from tank 1 and lift out of water
3. Start 1 min timer
4. Hold fish above tank, out of water
   ⇒ Cover net with divider to prevent fish jumping out

   End 1 min timer
5. Release fish back into tank, remove net
6. Start 10 min timer no.1

   At 2 min on no.1
7. Net all fish from tank 2 and lift out of water
8. Repeat 3-5
9. Start 10 min timer no.2

   At 2 min on no.2
10. Net all fish from tank 3 and lift out of water
11. Repeat 3-6

    Before end of no.1
12. Rinse all nets with system water

    At end of no.1
13. Repeat 2-12 twice more
14. Return all tanks to system

Clean-up (5 min)

1. Nets cleaned and hung up
2. Timers put away
3. Work space wiped

Chasing:

Equipment

- Small net x3
- Timer x3

Preparation (5 min)

1. Nets rinsed with system water
2. Work space cleared
3. Timers set to 5 min

Procedure (20 min)

1. Start with tank S1, then S2, end with S3
2. Remove home tank from system, remove lid
3. Start timer
4. Net in tank, chase fish
   ⇒ Keep net in mid-upper water level to minimise risk of jumping

   Timer end
5. Remove net, replace lid
6. Return tank to system
7. Repeat for other tanks

Clean-up (5 min)

1. Nets cleaned, hung up
2. Timers put away
3. Work space wiped

Low water:

Equipment

- Plastic tubing
- Timer
- White water container

Preparation (5 min)

1. Timer set for 2min
2. Container rinsed and set in stable position on floor

Procedure (15 min)

1. Start with tank S1, then S2, end with S3
2. Remove home tank from system and remove lid
3. Use air tubing to siphon water from tank into container until water reaches the level at which dorsal surface of fish is exposed
   ⇒ Press tube end into bottom corner of tank to avoid injuring fish
4. Remove and drain siphon into bucket
5. Start timer

   Timer end
6. Refill tank with water from container
   ⇒ Pour water slowly onto the sloped side of the tank
7. Return tank to system
8. Repeat for tanks 2 and 3

Clean-up (5 min)

1. Bucket and siphon cleaned and put away
2. Work surface wiped
3. Timer put away

Appendix C

Sampling

Schedule and procedures

Planned schedule:

Time	Task
11.00	
11.30	
12.00	Prep
12.30	
1.00	Briefing
1.30	#1 tanks
2.00	Reset
2.30	#2 tanks
3.00	Reset
3.30	#3 tanks
4.00	Debrief
4.30	Clean-up
5.00	
5.30	

Procedure

Euthanasia

Total 114 fish in 6 tanks

Equipment (per tank)

1. 2x breeding tank with inner removed, labelled to correspond with home tank
   ⇒ One will serve as euthanasia tank, the other will be used to separate the RNA sample fish for dissection (sampling tank)
2. Ice
3. Ice packs
4. Cold water
5. Thermometer
6. Tecniplast net, labelled to correspond with home tank
7. Small net, labelled to correspond with home tank and sample type
8. Timer
9. Mixing spoon/rod

Preparation (per tank)

1. Fill euthanasia tank to just over half-way with ice
2. Add cold water to cover ice
3. Mix until ice is fully submerged
4. Check temperature is below 4°C
5. Rinse net with system water
6. Set timer to 2 min
7. Repeat 1-5 for corresponding sampling tank

Procedure

One control and one treatment tank to be sampled simultaneously (n=38)

1. Remove home tank from system and place next to corresponding euthanasia tank
2. Net all fish together
3. Immediately transfer to euthanasia tank and release all fish
4. Gently mix slurry to ensure all fish are fully submerged
5. Start timer when all fish have lost equilibrium and no opercular movement can be seen

At end of timer

6. Transfer 6 fish to RNA sampling tank
7. Record time of death on sampling sheet

Dissection

RNA

Equipment (per person)

Dissection

- Paper towel
- Plastic spoon
- Latex gloves
- Forceps (160mm Rochester Pean straight, for attaching blades)
- Forceps (smooth, for handling body)
- Fine forceps (for handling brain)
- Scissors (curved)
- Scalpel handle (no. 3)
- Scalpel blade (no. 10)
- Wax dissecting pad
- Petri dish (35mm)
- PBS 1x solution (for floating fish if desired)
- Dissecting pins
- Dissecting microscope

Storage

- Tubes
- Tube rack
- Permanent marker

Cleaning

- Sharps bin
- Biological waste bin
- Plastics bin
- Wipes
- Isopropanol (spray bottle for wipes)
- Isopropanol (beaker/container for tools)
- RNAzap (spray bottle)

Preparation

1. Paper towels and spoons laid out next to sampling tank
2. Dissecting kit labelled and laid out
3. Isopropanol solution in container nearby
4. Blade on scalpel, placed in easily visible location
5. Bins lined and placed nearby
6. Tube rack on dry ice
7. Microscope turned on
8. Tubes labelled

Procedure

1. Remove single fish from sampling tank, place on paper towel
2. Blot body on towel using either spoon or forceps
   - ⇒ Handle body only, fins will rip easily
   - ⇒ If using spoon, scoop up in caudal direction to avoid snagging fins

3. Place body on wax, dorsal side up
4. Pin body to wax
5. Place body under microscope and adjust focus
6. Locate skull cap
7. Remove skull cap by scraping caudal end with scalpel in cranial direction
8. Cut off skull at cranial end if required
9. Cut caudal nerves, lift brain out with forceps
10. Cut optic nerves to remove brain
11. Place brain in storage tube
12. Check head cavity for any remaining brain tissue, particularly olfactory bulbs
13. Remove any remaining brain tissue and add to tube
14. Seal tube
15. Remove scalpel blade in sharps bin
16. Fill out spreadsheet
17. Unpin body and place in bin
    - ⇒ If petri dish used, empty into biological waste bin and place in plastics bin
18. Place all instruments in isopropanol solution
19. Wipe wax with isopropanol followed by RNAzap
20. Remove instruments and place on paper towel to dry
21. Place used paper towels and spoons in bin

DNA

Equipment (per person)

- Paper towel
- Plastic spoon
- Forceps (smooth, for handling body)
- Tubes
- Tube rack
- Permanent marker
- Dry ice

Preparation

1. Paper towels, spoons and forceps laid out next to sampling tank
2. Storage tubes
3. DNA tube rack labelled and placed on ice

Procedure

1. Remove single fish from sampling tank, place on paper towel
2. Use plastic spoon to roll body over paper towel to blot off excess water
3. Use forceps to place body in storage tube
4. Seal and label tube
5. Place tube in DNA tube rack

Cortisol

Equipment

- Paper towel
- Plastic spoon
- Forceps (smooth, for handling body)
- Tubes
- Tube rack
- Permanent marker
- Dry ice

Preparation

1. Paper towels, spoons and forceps laid out next to sampling tank
2. Storage tubes filled with preservation solution
3. Cortisol tube rack labelled and placed on ice

Procedure

1. Remove single fish from sampling tank, place on paper towel
2. Use plastic spoon to roll body over paper towel to blot off excess water
3. Use forceps to place body in storage tube
4. Seal and label tube
5. Place tube in cortisol tube rack

Storage

Labels

Storage tubes to be labelled with number from 1-114. Corresponding data sheet to identify each fish with sample number, tank identifier, followed by sample type, followed by initials of person dissecting.