DIETARY EFFECTS ON ADULT PERFORMANCE AND OXIDATIVE STRESS STATUS IN THREE-SPINED STICKLEBACKS

LYNDSEY A.E. STEWART

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CANDIDATE'S DECLARATION

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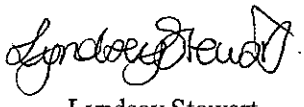

Lyndsey Stewart

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ABSTRACT

Nutritional conditions during early life can strongly influence the development of an organism in terms of immediate effects on early growth rates but also by shaping key life history traits later. After a period of nutritional deficit in early life, some animals have been found to accelerate their growth to compensate for the bad start once conditions have improved. There is experimental evidence demonstrating that compensatory growth can carry associated long-term costs, such as reduced locomotory capability and reduced investment in reproduction. Further, it has been proposed that oxidative stress may play an integral role in evoking these costs, as a result of a higher metabolic activity during compensatory growth. However, there is currently little literature available to attest this. Dietary-acquired antioxidants are proposed to support the endogenous antioxidant system in preventing oxidative stress and are therefore suggested to mediate life history trade-offs. In this thesis, resveratrol and carotenoids (potential antioxidants) were supplemented in the diets of three-spined sticklebacks *Gasterosteus aculeatus* in order to assess whether they were able to mitigate the negative effects associated with compensatory growth by reducing oxidative stress. Two important components of oxidative stress, antioxidant enzyme activity levels and oxidative damage were measured.

This thesis showed that food restrictions early in life resulted in a slowing of growth which was subsequently fully compensated for by acceleration in growth once food conditions were restored. Despite adopting different growth patterns, these fish achieved the same average size by sexual maturity as their control growth peers. However, compensatory growth was found to result in significant costs in later life including reduced cognitive and locomotor performance (Chapter 2). Additionally, it was demonstrated that food supplemented with resveratrol had some beneficial effect on cognitive performance in comparison with same-age controls (Chapter 2). However, resveratrol and carotenoid manipulations did not influence locomotor performance (Chapter 2). This result implies that dietary antioxidants were unable to offset the damage to swimming performance caused by compensatory growth, and that their generally beneficial effects in the diet do not affect all aspects of performance similarly, or to the same extent. Both dietary resveratrol and carotenoids influenced oxidative stress status (Chapter 3). Their role in the antioxidant system appears important and complex in that there were several statistical interactions between these dietary antioxidants and endogenous antioxidant enzyme levels. These enzymes play key roles in the defence of reactive oxygen species (Chapter 3). Fish

supplemented with a diet higher in carotenoids were found to have reduced levels of oxidative damage to proteins (Chapter 3). This result suggests that although carotenoids appear to be unimportant antioxidants for birds, these findings should not be generalised across all taxa.

Carotenoid and resveratrol availability influenced female reproductive investment (Chapter 3). Females on a diet supplemented with carotenoids had larger clutches suggesting that carotenoids played a positive role in their reproduction (Chapter 3). However, this was only apparent when the fish had not also been supplemented with resveratrol, suggesting that resveratrol may have imposed a detrimental effect on egg production (Chapter 3). Further, male reproductive investment in sexual ornamentation and nest building ability was also influenced by these dietary antioxidants in a similar fashion (Chapter 4: males fed a diet higher in carotenoids had significantly brighter throats at two crucial stages of the breeding season, while resveratrol had no positive effects on the intensity of the males' red throat signals). This is contrary to the so-called Red Herring hypothesis which suggests that carotenoid-based sexual signals advertise not the carotenoids themselves but other colourless antioxidants. In addition, males fed a diet lower in carotenoids took longer to both begin and complete nest building, whilst resveratrol had no influence on any aspects of nest building (Chapter 4).

The process of mate choice has been suggested to be costly in many species, since the assessment and comparison of potential mates is an energetically demanding process and hence likely to increase oxidative stress. Therefore, it was predicted that resveratrol would facilitate active mate choice in females through beneficial effects associated with its antioxidant properties. Indeed, females supplemented with resveratrol spent significantly more time associating with males than females that had not been fed resveratrol (Chapter 5). These female mate-choice experiments also demonstrated that resveratrol and compensatory growth did not impact male attractiveness (Chapter 5), suggesting that the females did not receive any alternative (and potentially important) mate cues mediated by resveratrol that were independent of the carotenoid-based signal.

There has been growing evidence in the literature demonstrating that resveratrol has neuroprotective properties, therefore reducing age-related reduction in cognitive performance, and this was also supported in the results of this thesis produced in Chapter 2. Chapter 6 investigated whether the supplementation of resveratrol and carotenoids

reduced the rate of ageing in three-spined sticklebacks in terms of exploratory and anxiety-related behaviour in an open field test, measured during early life and again in adulthood. However, in contrast to expectations, males that were not fed resveratrol had the greatest increase in exploratory activity across the experimental period (Chapter 6).

Overall, this thesis demonstrates that dietary manipulation with resveratrol (alongside carotenoids) affected numerous life history traits throughout the three-spined stickleback's lifespan. The evidence produced in this thesis strongly suggests that numerous aspects of an organism's performance can be affected by key nutrients, over and above calorie intake which is often misconceived as having unprecedented importance. These effects can be subtle in some scenarios and much more complex in others and they do not necessarily generalise terribly well across taxa. Moreover, by measuring oxidative stress status alongside later life performance in these fish, this thesis helps elucidate the precise roles these antioxidants have in influencing oxidative stress and alleviating the negative effects associated with compensatory growth.

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CHAPTER 1 – GENERAL INTRODUCTION

1.1 THESIS OVERVIEW

Nutritional ecology is at the forefront in understanding life history evolution. The feeding behaviours of organisms in terms of what they eat, when they eat, and how they allocate their food resources to different life functions contributes greatly towards the diverse range of life histories seen in the living world (Boggs 2009). Nutritional conditions, during early life in particular, can strongly influence the subsequent development of an organism in terms of immediate effects on early growth rates but also by shaping key life history traits in the long term (Metcalfe & Monaghan 2001). Both the quantity and the quality of the diet can be important. When considering the quantity of food, a period of food shortage often prompts a catch-up compensatory growth response once conditions have improved. However, a comprehensive meta-analysis across diverse taxa has recently affirmed the assumption that compensatory growth may carry associated costs (Hector & Nakagawa 2012). One such cost could be an increase in oxidative stress, as a result of this faster growth rate generating higher metabolic activity (Mangel & Munch 2005; Geiger et al. 2011). In turn, oxidative stress can negatively affect key traits related to fitness such as development, repair, maintenance, locomotor performance and reproduction (Lee et al. 2010; Metcalfe & Alonso-Alvarez 2010; Lee et al. 2012).

The effects of diet quality are more complex and difficult to evaluate, since there are multiple potential interactions between dietary components that may have positive or adverse effects on health and performance. For instance, resveratrol and carotenoids are dietary antioxidants which have been reported to have benefits which modulate life history trade-offs (Valenzano et al. 2006b; Orledge et al. 2012). Therefore, it would be expected that their presence in the diet would mitigate the negative effects of compensatory growth by reducing oxidative stress. However, although their antioxidant properties have been established *in vitro*, the literature on the antioxidant roles of carotenoids and resveratrol *in vivo* is both contradictory and confusing (Halliwell 2008; Yeum et al. 2009; Svensson & Wong 2011; Hector et al. 2012).

By means of experiments using three-spined sticklebacks *Gasterosteus aculeatus*, this thesis investigates links between nutritional ecology, resource allocation and life histories,

in particular the extent to which resveratrol, carotenoids and compensatory growth affect levels of oxidative stress and life history.

1.2 LIFE HISTORY TRADE-OFFS

A life history describes the life of an individual from its birth to its death in terms of its growth, maturation, reproduction and survival (Stearns 1992). Life history research attempts to account for the enormous variation found in life histories in the living world. This encompasses variation found among individuals, populations, species, and also across the diverse array of natural environments. For example, despite belonging to the same species, there are stark differences in the life history strategies of four geographically separate Pacific cod *Gadus macrocephalus* populations: southern stocks grow and mature much quicker but reach a smaller maximum size and live shorter lives than their northern equivalents (Ormseth & Norcross 2009). Their substantial life history variation represents adaptations to the characteristics of their different environments that have influenced the expression of particular traits (Ormseth & Norcross 2009). Evolutionary theory hypothesises that populations of the same species with different life histories, such as the Pacific cod, can maintain equal lifetime reproductive success through the existence of trade-offs among life history traits (Beverton 1987). This is the case with the Pacific cod, their adaptations have resulted in similar lifetime reproductive success across the four populations (Ormseth & Norcross 2009).

Life history trade-offs are the negative associations often found between life history traits within an organism as a result of resource limitations (Stearns 1989). For example, an optimal life history would be to commence reproduction at birth, produce an infinite number of offspring and live forever. However, this hypothetical organism - commonly referred to as a "Darwinian demon"- cannot exist as trait combinations are constrained by trade-offs (Roff 2002). The concept whereby fitness-related trait combinations are constrained by trade-offs has been of prominent interest in the field of evolutionary biology for over 80 years (Fisher 1930; Stearns 1992; Zera & Harshman 2001; Roff et al. 2002; Braendle et al. 2011).

1.3 RESOURCE ALLOCATION TRADE-OFFS

As energy is often a limited internal resource, its partitioning between life history traits is a fundamental example of how negative associations between traits can occur. The traditional "Y" model of resource allocation illustrates how two traits can be represented as the product of the amount of a critical resource acquired and the fraction of this resource allocated to each trait (de Jong & Van Noordwijk 1992). Therefore, when energy is limiting, an increase in energy investment to one trait results in a decreased allocation of energy to another trait in equal measure (James 1974). This is known as the "Principle of Allocation" (Cody 1966). Development in this research area has now recognised that although the "Y" model is a good starting point, it is more likely that multiple traits are involved in competing against one another for multiple constituents of a resource, rather than one constituent in particular (Boggs 2009). Different functions within an organism which often compete for limited internal resources such as energy include reproduction, growth, somatic maintenance and storage (Stearns 1992). For example, the "disposable soma theory" postulates that organisms will divide their resources between reproduction and somatic maintenance in an optimal way which is dependent on the environment in which they live, e.g. the risk of environmental hazards, such as predation, starvation and disease (Kirkwood 1993). Therefore, if an organism has high reproductive output over a short period of time, the theory predicts that it will have incurred greater damage to tissues and will die younger than an equivalent organism that delays reproduction and diverts its resources into maintaining and repairing its soma (Kirkwood 1977).

Wing polymorphism in *Gryllus* species (crickets) is a classic example of a trade-off that is hypothesised to involve the partitioning of internal resources between traits, in this case between flight capability and reproduction (Zera et al. 1998; Zera & Larsen 2001). The ovary weight (which is proportional to fecundity) in flightless morphs of *Gryllus* can be as much as 400% heavier in comparison with flight-capable morphs. However, this investment in fertility is costly as it is linked to a complete loss of flight capability in flightless morphs. Dorsal-longitudinal flight muscle weight and lipid flight-fuel can be reduced in flightless morphs by up to 40% and 30%, respectively (Zera et al. 1998; Zera & Larsen 2001). Therefore, resources in the flightless morph have been directed away from the development of flight apparatus and invested in reproductive tissues instead. The results of a number of feeding studies in *Gryllus* have suggested that the underlying

physiological mechanisms responsible for this trade-off are exacerbated when energy input is decreased (Zera et al. 1998; Zera & Brink 2000).

Recent attempts have been made to understand the physiological mechanisms underlying such trade-offs and it has been suggested that although energy appears to be the primary currency in most resource allocation models, there are also non-energetic aspects to consider (Dowling & Simmons 2009). After sufficient calorie intake has been achieved, the effects of the availability of other nutrients on the expression of life history traits can be much more complicated to infer. However, despite playing no role in providing energy, these additional nutrients are considered to play a key role in shaping life history traits. Dietary antioxidants in particular, can influence life history trade-offs as the expression of life history traits can be negatively affected by high levels of oxidative stress (Catoni et al. 2008).

1.4 OXIDATIVE STRESS MEDIATES LIFE HISTORY TRADE-OFFS

As discussed above, in evolutionary ecology, trade-offs are commonly thought of in terms of resource allocation. However, a second type of trade-off that is also suggested to influence life history traits is oxidative stress. Oxidative stress occurs within an organism when the production of reactive oxygen species (ROS) exceeds the ability of an organism to prevent this damage by means of their antioxidant defence system (Finkel & Holbrook 2000). Therefore, oxidative stress has been proposed to act as a mediator of life history trade-offs as this imbalance generates negative consequences for other fitness-related traits (Monaghan et al. 2009). ROS are a group of highly reactive oxygen-containing molecules such as hydrogen peroxide, hydroxyl radicals and superoxide anions. ROS are produced as by-products of oxidation-reduction (REDOX) reactions including during oxidative phosphorylation and reactions involved in pathogen defence (Dowling & Simmons 2009). In low levels, ROS are essential molecules for a wide variety of vital biological processes (Catoni et al. 2008). For example, around 10% of endogenous ROS produced in animal cells serve important physiological functions such as immune defence enhancement, detoxification and cell signalling (Droge 2002; Monaghan et al. 2009). However, the effects of ROS are dose dependent as at higher levels, ROS can increase oxidative stress. Oxidative stress can constrain the expression of a diverse range of life history traits throughout the entire lifespan of an individual and beyond to its offspring by influencing fecundity and fertility (Metcalfe & Alonso-Alvarez 2010). There are many possible

ecological factors that can influence oxidative stress in an individual. These include environmental conditions, developmental stage, and the levels of metabolic activity incurred by an individual throughout these life stages (Monaghan et al. 2009; Pamplona & Costantini 2011).

1.5 DIETARY ANTIOXIDANTS MEDIATE LIFE HISTORY TRADE-OFFS

An elaborate network of antioxidants including dietary antioxidants are considered to play a fundamental role in reducing oxidative stress and are therefore hypothesised to influence life history trade-offs (Catoni et al. 2008). Although a large number of studies in behavioural ecology have attempted to evaluate the significance of dietary antioxidants in determining life history trade-offs, there are still important aspects within this research area to investigate further. For example, more focus is required on the interactions of dietary antioxidants as it is more likely they are ingested in combination in the wild (Wang et al. 2011). Looking at particular interactions between different types of antioxidants is important as previous studies have found that antioxidants can interact either positively or negatively with one another and as a consequence, this may alter their physiological impacts (Catoni et al. 2008; Orledge et al. 2012; Skibsted 2012). For instance, antioxidant synergism has been demonstrated between β-carotene and astaxanthin for lipid oxidation in membranes (Liang et al. 2009). This is the product of these two carotenoids possessing different balances between donor and acceptor properties. When astaxanthin is anchored in the water/lipid interface of the membrane, it acts as an electron transfer bridge across the lipid phase which allows β-carotene to reduce the radical cation of astaxanthin (Liang et al. 2009; Skibsted 2012). Conversely, antioxidant antagonism in peroxidising liposomes has been demonstrated between β-carotene and four highly reducing green tea polyphenols (Song et al. 2011). There has been little investigation into interactions between plant polyphenols and carotenoids in comparison with interactions between carotenoids themselves. However, further study of this may allow us to gain a deeper understanding of the often disputed role of carotenoids in the literature (Skibsted 2012) In addition, most previous research has focused on the effects of dietary antioxidants on lifespan and effects on other crucial life history traits such as growth, fertility, senescence and the expression of sexually selected traits have attracted less attention.

Dietary antioxidants comprise a group of several hundred compounds but can be grouped into four classes: vitamin C (ascorbic acid), vitamin E (tocopherols and tocotrienols),

carotenoids (e.g. lutein and astaxanthin) and polyphenols such as resveratrol (Catoni et al. 2008). The group that has received the most attention from behavioural ecologists are the carotenoids: these are pigments and have been well-studied in a wide range of species including zebra finches (Blount & Matheson 2006), American goldfinches *Spinus tristis* (Navara & Hill 2003), guppies (Kodric-Brown & Nicoletto 2001) and three-spined sticklebacks (Pike et al. 2011). Carotenoids play a widespread role in sexual signalling in the animal kingdom. Across multiple species, there is a mating preference for highly elaborated carotenoid-based sexual signals (Milinski & Bakker 1990; Amundsen & Forsgren 2001; Kwiatkowski & Sullivan 2002). Alongside sexual signalling, carotenoids are proposed to serve a number of additional physiological roles, for example as immunostimulants, and as antioxidants as previously mentioned (McGraw & Ardia 2003; Chew & Park 2004; Yeum et al. 2009). However these alternative roles are highly debated (Hartley & Kennedy 2004; Costantini & Møller 2008).

The antioxidant properties of carotenoids are suggested to be associated with their role as free radical scavengers alongside their exceptional single oxygen quenching abilities (El-Agamey et al. 2004). It has been suggested that individuals with access to a sufficient supply of antioxidants such as carotenoids show slower age-related declines in performance after experiencing oxidative stress (Blount & Matheson 2006; Bertrand et al. 2006a). Dietary carotenoids have been suggested to play a protective role in defending against age-associated diseases including cardiovascular diseases and cancer. However, based on findings from some further studies, it is clear that carotenoids do not always play a protective role against free radicals (Bertrand et al. 2006a). Moreover, it has been suggested that although carotenoids exhibit antioxidant activity *in vitro* (Krinsky & Yeum 2003), this may not necessarily be their main biological role *in vivo* (Hartley & Kennedy 2004). Hartley & Kennedy (2004) suggest that dietary carotenoids may instead play a role in signalling the concentrations of other non-pigmentary antioxidant molecules which protect the carotenoids from oxidative attack and therefore make them available for sexual signalling (Hartley & Kennedy 2004; Bertrand et al. 2006b). An incomplete picture of an association between dietary carotenoids and antioxidant activity remains in the literature, and their importance as antioxidants has yet to be fully determined (Yeum et al. 2009; Pérez-Rodríguez et al. 2010). Nevertheless, carotenoid supplementation across a wide variety of species has been shown to improve many key traits including growth (Biard et al. 2006), survival (Chien & Shiau 2005; Pike et al. 2007a), parental care (Pike et al.

2007c), reproductive performance (Rosa et al. 2012) and the expression of sexual ornaments (Blount et al. 2003b; Smith et al. 2007).

1.6 DIETARY RESTRICTION, OXIDATIVE STRESS AND AGEING

It is considered that the production of ROS, together with the ability of organisms to act in response to oxidative damage, is intricately related to the rate of ageing and senescence. This concept was first proposed as *The Free Radical Theory of Ageing* in 1956 (Harman 1956). It has been hypothesised that metabolic rate could mediate the link between oxidative stress and the rate of ageing. That is, individuals with higher metabolic rates and rates of reproduction should age faster due to increased ROS production (Dowling & Simmons 2009). In keeping with this hypothesis, the catarina scallop *Argopecten ventricosus* has a high energy-intensive lifestyle with a maximum lifespan potential of only two years. This short lifespan is considered to be the result of its high metabolic and active lifestyle characterised by early fecundity, fast growth and high swimming activity that increases the accumulation rate of irreparable oxidative damage (Guerra et al. 2012). In comparison, the ocean quahog *Arctica islandica* lives as long as 400 years and has a low metabolic rate, late maturation and slow growth. This extremely long-lived clam has also been found to have significantly lower levels of oxidative damage to proteins in comparison with shorter lived bivalves such as the catarina scallop (Ungvari et al. 2011).

However, further recent evidence has begun to question the importance of the free-radical theory framework and its proposed links to mechanisms of ageing (Gems & Doonan 2009; Pérez et al. 2009; Speakman & Selman 2011). Predictions based on the free-radical theory suggest that an increase in antioxidant defences should decrease oxidative stress, subsequently reduce rate of ageing and increase lifespan (Finkel & Holbrook 2000; Poljsak 2011). However, some experimental work in mice for instance has not supported this theory, whereby overexpression of mitochondrial superoxide dismutase did not increase lifespan (Jang et al. 2009). Additionally, the longevity demonstrated in insulin receptor substrate1 null mice is not associated with increased tissue antioxidant protection or reduced oxidative damage (Page et al. 2013). Nor has the deletion of various antioxidant genes negatively impacted on lifespan in *C. elegans*, despite their presumed role in protecting against oxidative stress (Yang et al. 2007). In fact, there has been substantial evidence in opposition of the oxidative stress theory of ageing using multiple other species across a wide number of taxonomic groups (Buffenstein et al. 2008; Page et al. 2010;

Montgomery et al. 2012). Perhaps, the extremely long-lived naked mole-rat *Heterocephalus glaber* has contributed most greatly to the evidence disputing the direct link between reactive oxygen species production and lifespan (Rodriguez et al. 2011; Lewis et al. 2014). Naked mole-rats tolerate high levels of oxidative stress and have unremarkable antioxidant defences (Edrey et al. 2011). Regardless of this, and despite being of similar size and incurring comparable levels of ROS production to mice, they defy the traditional rodent lifespan curves and achieve an exceptional maximum lifespan of 35 years (Lewis et al. 2014). It has been suggested that cytoprotective mechanisms may help long-lived animals, such as the naked more-rat, deal with high levels of oxidative stress and this may underlie the reasons behind their long lifespans (Lewis et al. 2014).

Nonetheless, a substantial amount of evidence still exists in support of the free-radical theory of ageing and it has been suggested that perhaps using lifespan as a gold standard measure of the rate of ageing needs to be re-evaluated (Speakman & Selman 2011). It has been proposed that measuring lifespan to assess links between oxidative stress and ageing is an over-simplistic approach (Speakman & Selman 2011). Alternatively, it has been recommended that organism health in terms of the prevalence of age-related diseases may be more appropriate life-history criteria to use when evaluating the importance of the oxidative stress theory of ageing. There has also been an increasingly more common use of age-related markers such as locomotor and cognitive performance in assessing the role of oxidative stress in ageing (Valenzano et al. 2006a).

It has long been held in gerontology that dietary restriction (DR) can extend lifespan in a diverse range of taxa (McCay et al. 1939; McCay et al. 1943; Kaeberlein et al. 2006; Anderson & Weindruck 2010), through working as a hormesis agent. The hormesis hypothesis states an adaptive response mechanism is intrinsically provoked during DR, which allows the organism to cope with, potentially otherwise, adverse feeding conditions (Poljsak 2011). Outdated work suggests that the life extension ability of dietary restriction is linked to reductions in peroxidative stress through enhancing overall antioxidant capacity (Koizumi et al. 1987; Semsei et al. 1989; Lee & Yu 1991; Sohal & Weindruch 1996). For example, DR has been associated with a decline in lipid peroxidation and protein oxidative damage in fruit flies *Drosophila melanogaster* (Ward et al. 2005; Miwa et al. 2005). Paradoxically, more recent work has emphasized the importance of mitochondrial hormesis in mediating the DR effect, which is the activation of mitochondrial oxygen consumption in order to promote an increased production of ROS

(Ristow & Zarse 2010; Ristow & Schmeisser 2011). In low levels, these ROS have been found to serve as important cell signalling molecules which promote longevity by subsequently inducing ROS defence, through the activation of stress response pathways which ultimately reduces oxidative stress (Schulz et al. 2007; Woo & Shadel 2011).

DR has also been proposed to result in an adaptive shift in the allocation of resources from reproductive investment to somatic maintenance (Holliday 1989; Shanley & Kirkwood 2000). Long-term survival can then be enhanced through an increase in maintenance through investment in somatic defence and repair, which consequently postpones reproduction or reduces reproductive potential (Poizat et al. 1999). The seminal work of McCay and co-workers in 1935 showed that restricting food intake in rats caused a significant increase in lifespan (McCay et al. 1935). Up until recently, caloric restriction (energetic stress through restrictive feeding regimes) was thought to be a major, if not the sole dietary factor extending lifespan and reducing rate of ageing (Piper et al. 2005; Masoro 2006). However, more current studies have suggested that restrictions in the input of specific nutrients may also modulate lifespan and the rate of ageing. For example, independent of caloric content, lifespan was modulated by carbohydrate and protein manipulations in the diet of fruit flies *Drosophila melanogaster* (Skorupa et al. 2008) and field crickets *Teleogryllus commodus* (Maklakov et al. 2008). Most interestingly, protein restriction has been linked to significantly decreased mtDNA oxidative damage in rat liver suggesting that ROS may play an important part in mediating this trade-off between lifespan and reproductive fitness (Sanz et al. 2004; Sanz et al. 2006). Additionally, less oxidative damage to proteins and DNA was observed in yellow-legged gull chicks *Larus michahellis* after a short period of food restriction in comparison with chicks fed a plentiful supply of food (Noguera et al. 2011). These results, along with many others, uphold the hypothesis that mitochondrial hormesis may cause the lifespan extension caused by DR, provided that the restricted diet is manipulated above the threshold of malnutrition, relative to the model species in question (Ristow & Schmeisser 2011; Poljsak 2011) .

1.7 RESVERATROL AS A PROPOSED DR MIMETIC AND ANTIOXIDANT

Resveratrol is a natural polyphenolic compound produced by certain plants in response to pathogen invasion (Burns et al. 2002). Specifically, resveratrol is known to be produced by grapes, peanuts, soy and the Itadori plant *Polygonum cuspidatum* (Arichi et al. 1982; Sato et al. 1997; Sanders et al. 2000). Scientific interest in the biological activities of resveratrol

has existed for more than 30 years (Langcake & Pryce 1976). For example, resveratrol is considered to possess numerous health benefits such as anti-ageing, anticancer and neuroprotective properties which have been associated with its proposed antioxidant abilities (Fremont 2000; Corder et al. 2006; Pervaiz & Holme 2009; Qian et al. 2009). It has been found to extend lifespan by slowing the rate of ageing in the budding yeast *Saccharomyces cerevisiae* (Howitz et al. 2003), the nematode *Caenorhabditis elegans* (Viswanathan et al. 2005) and the fruit fly *Drosophila melanogaster* (Bauer et al. 2004; Wood et al. 2004). The exact underlying mechanisms involved in lifespan extension and reduced rate of ageing by resveratrol are a subject of controversy (Bass et al. 2007). However, resveratrol has been reported as a DR mimetic, whereby it is proposed to mimic the beneficial and health promoting effects of DR without having to actually restrict energy consumption (Poljsak 2011).

One suggestion is that resveratrol is a sirtuin-activating compound which slows metazoan ageing and prolongs lifespan by mechanisms related to DR (Wood et al. 2004). Sirtuins are a class of proteins which are required to be present and functioning for DR to extend lifespan (Lin et al. 2000; Wood et al. 2004). This proposed mechanism has been developed from initial findings that resveratrol promotes cell survival in the eukaryotic cells of *S. cerevisiae* by simulating Sir2 (a sirtuin protein) which then consequently increases DNA stability and prolongs the average lifespan of yeast by 70% (Howitz et al. 2003). This particular study also found that resveratrol had no effect on the lifespan of a Sir2 null mutant strain of *S. cerevisiae* (Howitz et al. 2003). This supported the proposed theory that resveratrol acts through the same pathway as DR by activating Sir2 (Howitz et al. 2003). The effects of resveratrol on the enzymatic activity of sirtuins have also been shown to prolong lifespan in multicellular animals including *D. melanogaster* and *C. elegans* by up to 14% and 29%, respectively (Wood et al. 2004). Resveratrol has also been found to increase the catalytic activity of human SIRT1 (a sirtuin protein) which consequently promotes cell survival, resulting in an extension of lifespan due to a reduced rate of ageing (Langley et al. 2002).

However, more recent studies have provided evidence which is inconsistent with this hypothesised mechanism that links resveratrol, sirtuin proteins and DR (Kaeberlein et al. 2005; Baur et al. 2006; Burnett et al. 2011). For example, the effects of resveratrol on lifespan were further tested in *D. melanogaster* in an attempt to reproduce the lifespan extension reported in the previous influential study by Wood et al. (2004). However, no

significant effects of resveratrol on longevity in *D. melanogaster* were found in seven independent trials, irrespective of the effort during the experimental design to replicate the original study as closely as possible (Bass et al. 2007). In addition, work by Burnett et al. (2011), found that resveratrol did not activate *d*Sir2 histone deacetylase. This finding contributes to other recent studies which have also disputed that resveratrol activates SIRT1 (Borra et al. 2005; Beher et al. 2009; Pacholec et al. 2010). Further to this, a Sir2 overexpression was not found to increase lifespan in both *C. elegans* and *Drosophila* (Burnett et al. 2011*)*. It was concluded that previously reported positive effects of sirtuin genes on lifespan were confounded by differences in genetic background and have challenged their validity, if not abolished these previous studies findings completely (Burnett et al. 2011). This new evidence suggests it is likely that an alternative mechanism is at play in lifespan extension using resveratrol as opposed to occurring through a mechanism related to DR.

One such potential mechanism is through its claimed antioxidant properties. There are a number of ways that resveratrol is proposed to act as an antioxidant including its ability to decrease mitochondrial ROS production, compete with the leakage of free radicals produced in co-enzyme Q (which is part of the electron transport chain that plays a critical role in the production of ATP), scavenge free radicals such as superoxide radicals, inhibit lipid peroxidation and also regulate the expression of antioxidant enzymes (Pervaiz & Holme 2009). For example, supplementation with resveratrol significantly ameliorated oxidative damage to DNA, increased enzymatic antioxidant status and reduced the extent of lipid peroxidation in the cancerous colons of male adult Wistar rats (Sengottuvelan et al. 2009). Conversely however, resveratrol (along with many other polyphenolic compounds) has been found to act as a pro-oxidant in certain circumstances (Ahmad et al. 2005; Halliwell 2008; Kovacic & Somanathan 2010). For example, resveratrol has been found to exhibit pro-oxidant effects on the heart, liver and kidney of male rats during daylight hours, in contrast to having antioxidant effects at night (Gadacha et al. 2009). This suggested a day/night rhythm of resveratrol in influencing redox balance which was measured by means of thiobarbituric acid reactive species (TBARS) (Gadacha et al. 2009).

Regardless, the relevance of these previous findings to pharmacological research on ageing in vertebrates such as humans is restricted by the fact that these invertebrate model species have completely different anatomical organizations (Terzibasi et al. 2007). However, most interestingly, both the life-extension ability of resveratrol and its ability to retard the onset

of a number of age-related markers have been discovered in a vertebrate, the short-lived fish, *Nothobranchius furzeri* (Valenzano et al. 2006b). In addition, another vertebrate study found that high calorie obese mice supplemented with resveratrol had reduced signs of age-related negative effects and longer lifespans than standard-fed mice (Baur et al. 2006). The beneficial effects of resveratrol in both *N. furzeri* and laboratory mice suggest that the anti-ageing effects of resveratrol described in yeast, fruit flies and nematode worms can be extended to vertebrates (Terzibasi et al. 2007). However, the overall conclusion of the "Resveratrol 2010" conference was that the published evidence was not sufficient enough to support the suggestions that resveratrol has beneficial effects on humans and that further research in vertebrates was required (Vang et al. 2011).

Although it can be postulated that resveratrol could modulate life history trade-offs through its ability to reduce oxidative stress, the hypothesis that resveratrol is an effective antioxidant requires more examination in animal models as they have received considerable less attention in comparison with *in vitro* studies (Baur & Sinclair 2006). Therefore, more exhaustive studies should be carried out *in vivo* to accompany the long string of *in vitro* studies that have been carried out in the past (Gülçin 2010; Holthoff et al. 2010; Escoté et al. 2012). In addition, studying the life history effects of resveratrol over the whole lifespan of an organism, in a context where animals face resource allocation trade-offs is imperative to understanding its effects in a realistic and evolutionarily relevant setting. Such knowledge is still required to assess reliably the potential capacity of resveratrol's function as an antioxidant and whether it can decrease the rate of senescence.

1.8 EARLY GROWTH AND THE COSTS OF COMPENSATION

Although DR is thought to extend lifespan by ultimately reducing oxidative stress through mitochondrial hormesis, compensatory growth is a widespread response commonly adopted by organisms after DR which can increase oxidative stress (Hector & Nakagawa 2012). Conditions during early life can induce profound oxidative challenges to young individuals due to an increase in metabolic rates required for growth during development (Monaghan et al. 2009). These oxidative challenges are particularly acute in animals that undergo a period of accelerated growth which occurs during periods of compensation. Compensatory growth usually occurs subsequently after a period of nutritional deficit in early life, when many organisms exhibit accelerated growth to compensate for this bad start once conditions have improved (Metcalfe & Monaghan 2001). For example, juvenile

green swordtails *Xiphophorus helleri* that were maintained on a restricted food ration were found to be significantly smaller at six months of age than the control group fed *ad libitum*. However, after being subsequently provided with access to *ad libitum* food, the group which had initially suffered a reduced growth rate were able to compensate for this period of deprivation and were no different in size to the control group by adulthood (Walling et al. 2007). Therefore these fish compensated for this period of food deprivation by modifying their growth trajectory in order to catch up in size when food was made available.

Compensatory growth has been found to affect the pattern of ageing. This is assumed to be partially due to a reduced investment in defences against oxidative stress as a consequence of limited resources, both during the initial period of deprivation and during the catch-up phase where the priority is the growth of new rather than the maintenance of old tissues. It has also been suggested to be due to an increased risk of physiological damage to molecules within the soma (e.g. DNA, RNA, lipids, proteins) as a result of higher metabolic activity during the period of compensatory growth (Mangel & Munch 2005; Inness & Metcalfe 2008). There is an abundance of examples in the literature, across a wide range of taxa, which suggest numerous long-term fitness consequences associated with this rapid growth in terms of development, repair, maintenance, locomotor performance and reproduction (Morgan & Metcalfe 2001; Robinson & Wardrop 2002; Álvarez & Metcalfe 2005; Lee et al. 2010; Auer et al. 2010b; Lee et al. 2012). For example, subsequently after a period of low winter food availability, wild king penguin chicks *Aptenodytes patagonicus* increased their growth rates to the detriment of their body maintenance and suffered greater oxidative damage than earlier-hatching chicks that had access to pre-winter food and that had not exhibited growth compensation (Geiger et al. 2011).

1.9 TRADE-OFF ASPECTS OF CAROTENOIDS AND RESVERATROL

This study investigated the potential antioxidant effects of carotenoids and resveratrol and their ability to modulate life histories, using the three-spined stickleback as a study system. The traits investigated were as follows:

Sexual signal investment - The relevance of carotenoid-based sexual signalling in sexual selection theory has become increasingly prominent within the field of behavioural

ecology in the last two decades (Catoni et al. 2008). Carotenoids cannot be synthesised within the body and can only be obtained through dietary means (Olson & Owens 1998). Therefore, internal trade-offs in the allocation of carotenoids to both signal expression and in alternative physiological functions can result when dietary carotenoid availability is limited (Chew & Park 2004; Pike et al. 2007c). For this reason, it is suggested that males that can afford to produce more elaborate carotenoid-dependent displays are signalling their enhanced ability to resist parasites, disease and oxidative stress and are hence predicted to live longer (Lozano 1994). It has been hypothesised that there should be a mating preference for males with the most colourful sexual ornaments as only high quality males should be able to afford a large investment of carotenoids in sexual signals (Bertrand et al. 2006b). Indeed, this has been found to be the case in numerous studies where females are attracted to more highly elaborated males (McGraw & Ardia 2003; Pike et al. 2007a). This concept underpins Zahavi's handicap principle that states that strategic investment into sexual signalling comes at a cost, and it is this cost that maintains signal honesty (Zahavi 1975).

However, the honesty of animal communication remains a highly debated topic in the literature (Szamando 2011). For example, a male's sexual signal may be altered as a consequence of the negative effects associated with compensatory growth, or a male may attempt to overcompensate for this by attempting to maintain his sexual signal to the detriment of his health (Lindström et al. 2009). A recent study found that a male's sexual ornamentation was negatively affected by compensatory growth in three-spined sticklebacks (Lee et al. 2012). Therefore, this thesis investigated this further by examining the potentially positive effects of nutrient supplementation on the predicted negative effects of compensatory growth on investment in sexual signals.

Reproductive investment - "The cost of reproduction represents one of the most fundamental of life history trade-offs" (Dowling & Simmons 2009). The potential mechanisms underlying reproductive trade-offs such as survival versus reproduction, number versus size of offspring and current versus future reproduction are reviewed in detail in Edward & Chapman (2011). When resources are in short supply an organism may become unable to meet all the demands placed upon it (Edward & Chapman 2011). As reproduction is an energetically expensive activity, it often becomes relatively neglected in favour of other competing functions (Roff 2002). For example, it has been found in parasitic wasps that when internal resources are limited, allocation to maintenance and

storage is prioritised over allocation to reproduction (Wajnberg et al. 2012). In three-spined sticklebacks, reproduction imposes considerable expenditure for the males in particular (Cubillos & Guderley 2000). Elevated metabolic rate during activities such as courtship behaviour and paternal care activities including nest construction, fanning and defence of fertilised eggs can all increase the susceptibility of the males to oxidative stress. Carotenoid availability has previously been found to influence diverse aspects of the reproductive performance of male three-spined sticklebacks including their attractiveness to females (Pike et al. 2007a), their ability to hatch eggs (Pike et al. 2007c) and their ability to maintain reproductive activities through the breeding season (Pike et al. 2010). For these reasons, reproductive investment was an ideal life history trait to investigate in this study. Also, resveratrol has been found to increase longevity without affecting fecundity (Wood et al. 2004; Chandrashekara & Shakarad 2011). This observation is counter to the presumed fecundity vs. survival trade-off in life history evolution. Therefore, further investigation is required into the effects of resveratrol on parental investment.

Age-related declines in life functions - A link between compensatory growth in early life and accelerated rate of ageing has been established in a number of age-related traits and also in survival (Monaghan & Metcalfe 2003). This thesis investigated the extent to which carotenoids and resveratrol modulated the negative association found between compensatory growth and adult performance by measuring a number of age-related markers. These included swimming performance, cognitive performance and performance in an open-field test. Open-field exploration behaviours have been shown to have an age-dependent decline in rodents, fish and in a primate (Valenzano et al. 2006b; Abreu et al. 2011; Dal-pan et al. 2011). Locomotor performance has been found to exhibit senescence (Lucas-Sánchez et al. 2011), and this age-related decline has been found to be hastened by compensatory growth in sticklebacks (Álvarez & Metcalfe 2005; Lee et al. 2010; Pike et al. 2010b). Swimming performance was therefore an ideal trait to investigate, when assessing whether carotenoids and resveratrol could ameliorate these negative effects of growth rate on the rate of senescence.

Age-related reductions in cognitive performance can be quantified by operant learning and can be measured using a shuttle box protocol which is based on an active avoidance paradigm (Horner et al. 1961; Pradel et al. 1999). The shuttle box adaptation which was implemented in the present study has also been used in two previous studies determining whether resveratrol delays the age-dependent decay of cognitive performances in

Nothobranchius furzeri and *Nothobranchius guentheri* (Valenzano et al. 2006b; Yu & Li 2012). In these previous studies, old control fishes had a success rate of 42% and 43% in the shuttle box trials, respectively. However fish of the same age that had been fed with a supplement of resveratrol reached a success rate of 74% and 67%, respectively, suggesting that resveratrol slows the age-dependent decay of cognitive performance (Valenzano et al. 2006b; Yu & Li 2012). The present study aimed to test the effects of resveratrol in combination with carotenoids on cognitive decay in three-spined sticklebacks.

1.10 THREE-SPINED STICKLEBACKS AS A STUDY SPECIES

Three-spined sticklebacks are a species complex encompassing thousands of phenotypically diverse populations which differ in terms of their life histories (Bell & Foster 1994). These populations are widely distributed throughout the northern hemisphere and occur in both marine and freshwater habitats (Bell & Foster 1994). The present study used an annual riverine population from western Scotland. Three-spined sticklebacks have distinct breeding characteristics. Males develop a carotenoid-based red nuptial throat colouration when coming into reproductive condition (Östlund-Nilsson et al. 2007). As carotenoids can be limited in the diet there can be variability in the expression of this sexual signal, across different times in the reproductive cycle and also across individuals (Wedekind et al. 1998; Barber et al. 2000). Male three-spined sticklebacks construct nests at the onset of the breeding season in order to attract gravid females to the nest to spawn. The males then fertilise the eggs and provide all subsequent parental care. The nest is usually built on a sandy substratum with filamentous algae and other vegetation which is "glued" together with a kidney excretion containing a glycoprotein called spiggin (Wootton 1976). Alongside the red nuptial colouration, nest structure is also thought to act as a quality-revealing ornament for the females (Barber et al. 2001).

There is a wealth of background information on the biology of three-spined sticklebacks from both an evolutionary and behavioural perspective (Bell & Foster 1994; Östlund-Nilsson et al. 2007; Wootton 2009). Three-spined sticklebacks were therefore an ideal study species as they are characterised by numerous well-documented behaviours which related to the experimental aims of this study. For instance, female sticklebacks show mate choice preferences for redder males (Milinski & Bakker 1990) and previous studies have demonstrated the effects of carotenoids on life history patterns such as their role in trade-offs between sexual signalling and somatic maintenance (Pike et al. 2007a). In addition to

this, the effects of early growth trajectories (including compensatory growth) on adult performance and lifespan have also been well studied (Álvarez & Metcalfe 2005; Inness & Metcalfe 2008; Lee et al. 2010; Lee et al. 2012). Given their short lifespan, three-spined sticklebacks were also an ideal animal model for studying the effects of dietary manipulations on age-related markers.

1.11 AIMS OF THESIS

This thesis is based on results from a series of experimental investigations in which individuals were faced with resource allocation trade-offs from early life. Most previous life history experiments investigating the effects of resveratrol on animal models have been over-simplistic in that the animals have been given an unlimited access to food resources. This would therefore prohibit the observation of any trade-offs that may occur in the wild where resources are often limited. In these current experiments, individuals were subjected to a compensatory growth regime by restricting food initially and then uplifting the restriction in order to induce oxidative stress. These experiments then investigated the proposed antioxidant effects of two dietary supplementations, resveratrol and carotenoid content, on mitigating the negative effects associated with compensatory growth by measuring subsequent adult performance and later-life oxidative stress status. Numerous life history traits were measured, including reproductive investment, sexual signal investment and senescence by measuring a number of age-related markers in later life. These laboratory experiments also used a combination of different measurements to measure oxidative stress effectively, since the interpretation of many previous studies have been restricted by limited components of oxidative stress having been measured.

The objectives of this thesis were to address the following questions:

- Does compensatory growth induced by early restrictions in food availability affect two age-related markers, namely later-life cognitive and swimming performance? If so, to what extent are these negative effects of compensatory growth alleviated by the supplementation of resveratrol and carotenoids in the diet? (Chapter 2)

- Does resveratrol and carotenoid supplementation reduce oxidative stress induced by compensatory growth in both male and female three-spined sticklebacks? If this supplementation does indeed reduce oxidative stress in females, is this reflected in

a) her egg clutch investment and b) her egg quality in terms of the antioxidant capacity of her unfertilised eggs? (Chapter 3)

- Does compensatory growth induced by early restrictions in food availability elicit a trade-off between reproductive investment and somatic maintenance (measured in terms of oxidative stress status)? If so, to what extent does the supplementation of resveratrol and carotenoids in the diet affect a) the strength of the male's sexual signal and b) his nest building performance? (Chapter 4)

- Does a compensatory growth regime reduce a male's sexual attractiveness? If so, does the supplementation of resveratrol alleviate the negative effects associated with compensatory growth and subsequently improve male attractiveness in terms of female mating preference? (Chapter 5)

- Does the supplementation of resveratrol and carotenoids reduce rate of ageing in terms of exploratory and anxiety-related behaviour in an open field test? (Chapter 6)

The thesis is then concluded with a general discussion in which the results of these experiments are related to previous findings and potential future directions are identified. In addition, any problems and implications associated with the experiments undertaken in this thesis are highlighted (Chapter 7).

CHAPTER 2 – RESVERATROL AND CAROTENOIDS MEDIATE TRADE-OFF BETWEEN GROWTH RATE AND LATER-LIFE COGNITIVE BUT NOT LOCOMOTOR PERFORMANCE

2.1 ABSTRACT

This chapter investigated how compensatory growth affected subsequent cognitive and locomotor performance, and how any such effects were influenced by the availability of carotenoids and resveratrol in the diet. Using juvenile three-spined sticklebacks *Gasterosteus aculeatus*, this chapter shows that a short period of food restriction in early life had effects on the growth trajectory of the fish during the food restriction period itself but also on the subsequent growth trajectories up until nine weeks after this food restriction had been uplifted. This compensatory growth phase allowed these fish to achieve the same average body size by sexual maturity as their steadily-grown peers. However, achieving this body size came at a cost to both cognitive performance and swimming performance in adulthood. Interestingly, this chapter demonstrates that the availability of resveratrol in the diet led to an improved performance early in the cognitive performance task, but this was not maintained throughout the duration of the experiment. It was perhaps not surprising to find that dietary supplementation of resveratrol and carotenoids had no effect on swimming performance, due to the lack of obvious benefits they had on the antioxidant system. There was an observed downregulation in the endogenous antioxidant superoxide dismutase in the resveratrol-fed fish which may suggest that resveratrol influenced endogenous intracellular defence. However, there were no effects of the dietary supplements on GPx activity or protein carbonyl levels. Overall, these results show that the costs of compensatory growth are apparent well beyond the active period of catching up in size. However, these costs can be alleviated to some degree by the availability of key nutrients in the diet, such as resveratrol, which appears to exhibit some neuroprotection.

2.2 INTRODUCTION

2.2.1 The costs of compensatory growth and the role of dietary antioxidants

Trade-offs in life history traits such as between growth and ageing are postulated to be influenced by the diversity, availability and interactions of dietary antioxidants (Catoni et al. 2008). Ageing results from a lack of investment in particular maintenance traits which

causes malfunctioning of cellular and organismal functions over time (Finkel & Holbrook 2000). Therefore, antioxidant protection against oxidative damage is essential for organism function (Pamplona & Costantini 2011). Faster growth rates such as undergoing growth compensation are hypothesised to increase oxidative stress as a higher rate of reactive oxygen species (ROS) production accompanies greater cellular activity (Monaghan et al. 2009).

The benefits of undergoing compensatory growth are linked to the individual being able to achieve its full growth potential. For example, the benefits of a larger adult size include a reduced predation rate, a wider range of prey sizes that can be exploited and increased fecundity (Blanckenhorn 2005). These benefits have been mirrored in a simulation study using three-spined sticklebacks *Gasterosteus aculeatus*. The study evaluated the consequences for a population which failed to exhibit compensatory growth following a period of inadequate food supply (Wootton 2004). The simulations illustrated that three-spined sticklebacks that did not mount a compensatory growth response would experience adverse effects on fecundity, have a reduced attainable prey size and a possible higher rate of mortality (Wootton 2004).

However, growth rates during compensation exceed the growth rates of continuously-fed fish, which indicates that continuously-fed fish grow at a rate which is normally lower than what is maximally possible (Metcalfe & Monaghan 2001; Dmitriew 2011). This suggests that individuals are able to adjust their growth rates to some extent. Therefore, this has begged the question in a long list of compensatory growth studies (Hector & Nakagawa 2012): if some individuals are able to grow at maximal rates, why do all individuals not do so? The widely accepted explanation for this is that compensatory growth carries associated costs and that trade-offs exist between this faster growth rate and key fitness traits (Arendt 1997; Ali et al. 2003; Álvarez 2011). These costs may not often be apparent until much later in life (Metcalfe & Monaghan 2001). For example, juvenile salmon *Salmo salar* subjected to a short-lived autumnal food shortage exhibited compensatory growth once feeding conditions had improved again and managed to fully restore their lipid reserves in preparation for winter, relative to controls (Morgan & Metcalfe 2001). However, it was not until months after the compensation period had ended that the long-term negative effects of this compensatory growth response became evident. By spring, their growth rates were impaired, their lipid reserves were severely reduced and this

consequently led to a lower incidence of males reaching sexual maturation that year (Morgan & Metcalfe 2001).

Although individuals that have exhibited full compensatory growth may be indistinguishable in their external appearance from conspecifics that have grown steadily, less obvious fitness components such as oxidative stress status, cognitive performance and locomotor performance have been found to be adversely affected by compensatory growth (Álvarez & Metcalfe 2005; Fisher et al. 2006; Álvarez & Metcalfe 2007; Geiger et al. 2011; Dmitriew 2011).

Previous research has consistently found that after a period of nutritional deficit during development, three-spined sticklebacks accelerate their growth to compensate for this setback once conditions have improved (Ali et al. 1998; Zhu et al. 2001; Zhu et al. 2003; Álvarez & Metcalfe 2005; Inness & Metcalfe 2008; Lee et al. 2010). Therefore, three-spined sticklebacks exhibit a compensatory growth response which allows individuals to reach the same size-for-age as conspecifics that have not experienced a nutritional deficit (Álvarez & Metcalfe 2005). In the present study later-life oxidative stress status was measured in order to determine the extent to which resveratrol, a polyphenol, (alone and together with two carotenoids, lutein and astaxanthin) can control and mitigate the production of excess ROS produced during compensatory growth. Such knowledge is required to assess reliably the potential capacity of both carotenoids and resveratrol's function as antioxidants. It is important to determine the antioxidant function of carotenoids in combination with resveratrol as strong synergistic interactions between certain dietary antioxidants have often been found (Mortensen et al. 2001; Amorati et al. 2002; Frank 2005). Such interactions can either diminish any antioxidant properties or they may produce an additive antioxidant effect (Catoni et al. 2008). For example, a recent study found that 20 single dietary supplements had no antioxidant effect on the rotifer *Brachionus manjavacas* (Snell et al. 2012). However, seven two-way combinations of these dietary supplements produced positive antioxidant effects (Snell et al. 2012).

2.2.2 Cognitive performance as an age-related marker

Evidence from studies in several species including humans, rodents and songbirds, have established that poor nutrition in early life can have negative effects on mental development and consequently cognitive ability (Olson & Mello 2010; Zainuddin &

Thuret 2012). However, links between the role of compensatory growth in particular (which can follow a period of poor early nutrition), have not been so well studied. Although the evidence is limited, compensatory growth has been found to impair adult cognitive performance in zebra finches *Taeniopygia guttata* (Fisher et al. 2006). The ability to perform in a simple task in adulthood was negatively associated with the degree of growth compensation that had been exhibited by the birds, after a reduction in food supply during their first 20 days of life (Fisher et al. 2006). The present study aimed to determine whether a similar effect was found in other taxa, and whether carotenoids and resveratrol were able to mediate any such growth-performance trade-off. This may be particularly feasible in the case of resveratrol as alongside its reported antioxidant properties, there is increasing empirical evidence of its neuroprotective properties (Sebai et al. 2009; Robb & Stuart 2010; Fukui et al. 2010).

Age-related reductions in cognitive performance can be quantified by operant learning in an active avoidance paradigm using devices such as a shuttle box (a protocol originally developed for rodents but since adapted for animals such as goldfish and zebrafish (Warner 1932; Horner et al. 1961; Pradel et al. 1999)). Both pass rate and escape latency can be used as reliable indicators of learning in this active avoidance paradigm (Xu & Goetz 2012). The shuttle box has been previously used by Valenzano et al. (2006) and Yu et al. (2012), who both showed that resveratrol delayed the onset of age-dependent decay in cognitive performance in the turquoise killifish *Nothobranchius furzeri* and its close relative *Nothobranchius guentheri,* respectively. However, there are drawbacks associated with these two studies. For instance, the experimental fish were given a regular and plentiful supply of food throughout their entire lifespan. Therefore, it remains unknown how well resveratrol can modulate trade-offs in resource allocation, as trade-offs will not exist when resources are in plentiful supply (Van Noordwijk & de Jong 1986; Stearns 1989). These fish had also not been subjected to experimental manipulations to increase their oxidative stress. Therefore, conclusions cannot be drawn with regard to the extent to which the antioxidant capacities of resveratrol mediate trade-offs. However, the present study aims to determine how well resveratrol mediates trade-offs when fish have undergone compensatory growth, an energetically expensive activity which may promote oxidative stress.

The aim of the cognitive performance component of the present study was to investigate resveratrol's ability (alone and in combination with a high or lose dose of carotenoids) in

mediating the predicted negative effects associated with compensatory growth on cognitive performance, under more natural settings where the animals faced resource allocation trade-offs.

2.2.3 Swimming performance as an age-related marker

Compensatory growth is also known to impair locomotor performance (Álvarez & Metcalfe 2005; Lee et al. 2010). A potential cause for this is suggested to be the increased levels of oxidative damage to important muscles that are required in locomotion (Lee et al. 2010). This seems plausible, since a higher rate of cell division and free radical damage has been associated with rapid growth (Alonso-Alvarez et al. 2007; Geiger et al. 2011; Almroth et al. 2012). Age-related declines in locomotor efficiency have been used previously as a marker for neuromuscular decay in guppies *Poecilia reticulata* and killifish (Reznick et al. 2004; Valenzano et al. 2006b). Also, reductions in locomotor activity have been associated with accumulated oxidative damage to lipids in the Korthaus' killifish *Nothobranchius korthausae* (Lucas-Sánchez et al. 2011). These findings suggest that swimming performance could be used as a reliable age-related marker in the present study.

Previous studies investigating the impact of compensatory growth on swimming performance in three-spined sticklebacks have primarily focussed on the effect of a nutritional deficit in terms of its quantity rather than quality (Inness & Metcalfe 2008). However, it has now been recognised that, alongside the total amount of food (and hence overall energy intake), the effects of a shortage of specific dietary antioxidants may also be important (Pike et al. 2010b). For example, dietary carotenoid intake has been found to slow the age-related decline in swimming performance in three-spined sticklebacks in a study where sustained swimming performance was used as an indicator of locomotor senescence (Pike et al. 2010b). The study suggested that dietary carotenoids played an antioxidant role and offset the increased rate of senescence induced by an increased reproductive effort (which is an energetically expensive activity). A separate study found that resveratrol supplements helped to retard reductions in locomotor activity with age in *Nothobranchius furzeri* (Valenzano et al. 2006b). However, these fish were not manipulated under conditions that would increase oxidative stress. Nevertheless, these findings suggest that carotenoids and resveratrol may play a role in reducing the negative effects associated with other energetically costly activities that increase ROS production, such as compensatory growth (Valenzano et al. 2006b; Pike et al. 2010b).

This aim of the swimming trials in the present study was to investigate how compensatory growth affected later-life swimming performance, and whether dietary supplements of resveratrol and carotenoids reduced the predicted decline of sustained swimming performance by limiting the damaging effects of ROS that are associated with compensatory growth.

The experiments will help determine whether resveratrol (alone and together with carotenoids) can decrease the rate of senescence in three-spined sticklebacks, using both mental and physical assays of performance. The same source population of three-spined sticklebacks used in the present study have been previously well studied with regard to their growth, performance and lifespan (Inness & Metcalfe 2008; Lee et al. 2010; Lee et al. 2013). For instance, control growth three-spined sticklebacks from the same source have been found to live a median lifespan of 761 days in ambient photoperiod conditions, while those that have underwent a compensatory growth regime lived a median lifespan of only 651 days (Lee et al. 2013). In the present study, cognitive performance, swimming performance and oxidative stress status were measured in the three-spined sticklebacks at day 239, day 253 and day 281 in relation to the logistics used to assess lifespan in Lee et al. (2013) in the same source population. Therefore, in the present study the time points of the three aforementioned assays are regarded as a stage "later in life" as opposed to "late life" in the three-spined sticklebacks.

2.3 METHODS

2.3.1 Source of fish and rearing conditions

A total of 360 juvenile (based on body length) three-spined sticklebacks were collected from the River Endrick, Stirlingshire, Scotland (lat 56°04' N, long 4°23'W) using dip nets and minnow traps between 23rd March and 4th April 2010. All fish were caught from two nearby localities along the river in order to reduce potential confounding factors such as differences in carotenoid availability within their environment, prior to their diet being manipulated in this study.

Following capture the underyearling fish were transported to the University of Glasgow and transferred to acclimatization aquaria (45-L and density 3 fish L^{-1}). Fish were fed *ad libitum* (i.e. 10% of body mass/day) with defrosted chironomid larvae until the 29th June

2010 when the experiment commenced. The temperature and the photoperiod were adjusted each week to match the mean ambient conditions at the source river for that time of year. The photoperiod was achieved using fluorescent lighting and controlled by electronic timers. Tanks were inspected daily in order to monitor mortality rates throughout the experiment.

On the 29th June 2010, all 360 fish were anaesthetised and measured for standard length (the length from the top of the snout to the caudal peduncle ($\pm$0.01 mm)) and wet mass ($\pm$0.001 g). All 360 fish were then randomly assigned in groups of 15 into 24 separate 45-L tanks (58 × 38 × 26 cm). Each tank was provided with aeration, an individual filter, a gravel substrate and four artificial plants (to promote environmental enrichment, to reduce stress and also to provide refuges for the fish). Each tank was given a 25% water change each week (11.25L) until the end of the experiment. Salt was added at a concentration of 2g L^{-1} of tank water to prevent the risk of whitespot *Ichthyophthirius multifiliis* infection.

The fish were re-measured for length and mass regularly throughout the experiment. All fish were starved for 24 hours prior to taking their measurements, to prevent variation in stomach contents influencing measured fish mass. On 20th September 2010 three fish from each of the 24 tanks were culled for oxidative stress measurements in order to investigate whether resveratrol and carotenoid availability influenced oxidative stress status, soon after the completion of compensatory growth. However, all of these fish samples were unfortunately involved in a freezer malfunction and had to be excluded from any oxidative stress analyses as a subsequent degradation test proved them to be unreliable.

On 27th September 2010, the remaining fish were transferred to smaller tanks (33 × 18 ×19 cm) and sorted into groups of three. Individuals could then be identified for the remainder of the experiment on the basis of their size. All experiments were performed under licence from the UK Home Office (PIL 60/12423).

2.3.2 Growth manipulations

The experiment consisted of 8 feeding treatments, defined in relation to both growth regime and dietary supplementation. A more detailed description of the 8 feeding treatments that began on the 29th June 2010 can be found in Table 2.1. Twenty-four tanks of fifteen fish were randomly and evenly assigned between the compensatory (C) growth

regime and the *ad libitum* (A) growth regimes. The A growth regime fish received a regular and sufficient supply of food, delivered once per day, throughout the whole experiment so that their growth would be unrestricted. They received 10% of their body mass per day in chironomid larvae as it has been previously shown that three-spined sticklebacks produce maximum growth rates at this ration (Allen & Wootton 1982).

The C growth regime ration was set to keep their growth rates close to zero during Period 1 (Table 2.1); these fish were fed 2% of their initial body mass per day in chironomid larvae. This feeding ration has been used successfully in previous compensatory growth studies using three-spined sticklebacks (Álvarez & Metcalfe 2005; Inness & Metcalfe 2008). In this experiment, the restricted food ration was applied for a seven week period (Period 1); by this time point the C growth regime fish were significantly smaller in size in comparison with the A growth regime fish. This restricted ration was then uplifted on the 19th August 2010, after which point the C growth regime fish received a regular and plentiful supply of food equivalent to the A growth regime ration for the remaining experimental period to induce compensatory growth (Period 2) (Table 2.1).

2.3.3 Dietary supplement manipulations

In order to investigate whether resveratrol and carotenoid availability influenced later-life performance and oxidative stress status, the twelve tanks belonging to each of the C and A growth regimes described above were further randomly assigned into three replicate tanks for each of the four dietary supplement manipulations. These tanks were defined in relation to both resveratrol (present or absent) and carotenoid supplementation (high or low).

The fish in the high carotenoid and resveratrol (HR) treatment group received a high dose of carotenoids (equal quantities of lutein and astaxanthin at a total concentration of 200µg carotenoids g^{-1} of food) and a dose of resveratrol (415µg g^{-1} of food). The high carotenoid (HN) manipulation received the same high dose of carotenoids and no resveratrol. The low carotenoid and resveratrol (LR) manipulation received a low dose of carotenoids (equal quantities of lutein and astaxanthin at a total concentration of 10µg carotenoids g^{-1} of food) and a dose of resveratrol (415µg g^{-1} of food). The low carotenoid (LN) manipulation received the same low dose of carotenoids and no resveratrol.

The high and low carotenoid doses correspond to the lower and upper limits of carotenoid concentrations used in previous experiments using three-spined sticklebacks (Pike et al. 2007c; Pike et al. 2010a). Lutein and astaxanthin are both found naturally in the diet of this population of sticklebacks collected from the River Endrick (T.W. Pike, unpublished data). These particular carotenoids were manipulated in the present study as it has been previously found that the red colouration of the three-spined stickleback's nuptial throat area is a multiple signal produced by both astaxanthin and lutein (Wedekind et al. 1998).

At time of writing, *Nothobranchius furzeri* and *Nothobranchius guentheri* have been the only fish species fed a diet supplemented with resveratrol (Valenzano et al. 2006b; Yu & Li 2012). The dose of resveratrol chosen in this experiment was comparable to the doses successfully used in these previous studies. During Period 1, fish on the A growth regime received five times as much food as those on the C growth regime. For this reason, the carotenoid and resveratrol concentrations in the C growth food were multiplied by five. This was carried out in order to mitigate variation in the concentrations of carotenoid and resveratrol supplements received by the fish in the different growth regimes. Therefore, during Period 1 for fish in the C growth regime a low dose of carotenoids was defined as a concentration of 50µg carotenoids g^{-1} of food, a high dose of carotenoids was a concentration of 1000µg carotenoids g^{-1} of food and a dose of resveratrol was 2075µg of resveratrol g^{-1} of food. This adjustment prevented growth regime from confounding the carotenoid and resveratrol concentrations received by the fish.

Thus, overall commencing this experiment there were 8 feeding treatments (2 growth regimes × 2 resveratrol regimes × 2 carotenoid regimes), each with 3 replicate tanks containing 15 fish. This allowed evaluation of the effect of dietary restriction-induced compensatory growth on later-life cognitive performance, swimming performance and oxidative stress status (Figure 2.1). In addition, it could also be determined whether dietary supplements of resveratrol and carotenoids influenced any of these responses.

Table 2.1 Description of the eight feeding treatments. Following the seven week growth manipulation period (Period 1), all fish were then fed *ad libitum* for the remainder of the experiment in order to induce compensatory growth in the C growth regime (Period 2). Three replicate tanks of 15 fish were assigned to each treatment group.

Group	Growth regime		No. of tanks	Dietary supplementation
	Period 1	Period 2		
AHR	*Ad libitum*	*Ad libitum*	3	High carotenoid and resveratrol
AHN	*Ad libitum*	*Ad libitum*	3	High carotenoid no resveratrol
ALR	*Ad libitum*	*Ad libitum*	3	Low carotenoid and resveratrol
ALN	*Ad libitum*	*Ad libitum*	3	Low carotenoid no resveratrol
CHR	Compensatory	*Ad libitum*	3	High carotenoid and resveratrol
CHN	Compensatory	*Ad libitum*	3	High carotenoid no resveratrol
CLR	Compensatory	*Ad libitum*	3	Low carotenoid and resveratrol
CLN	Compensatory	*Ad libitum*	3	Low carotenoid no resveratrol

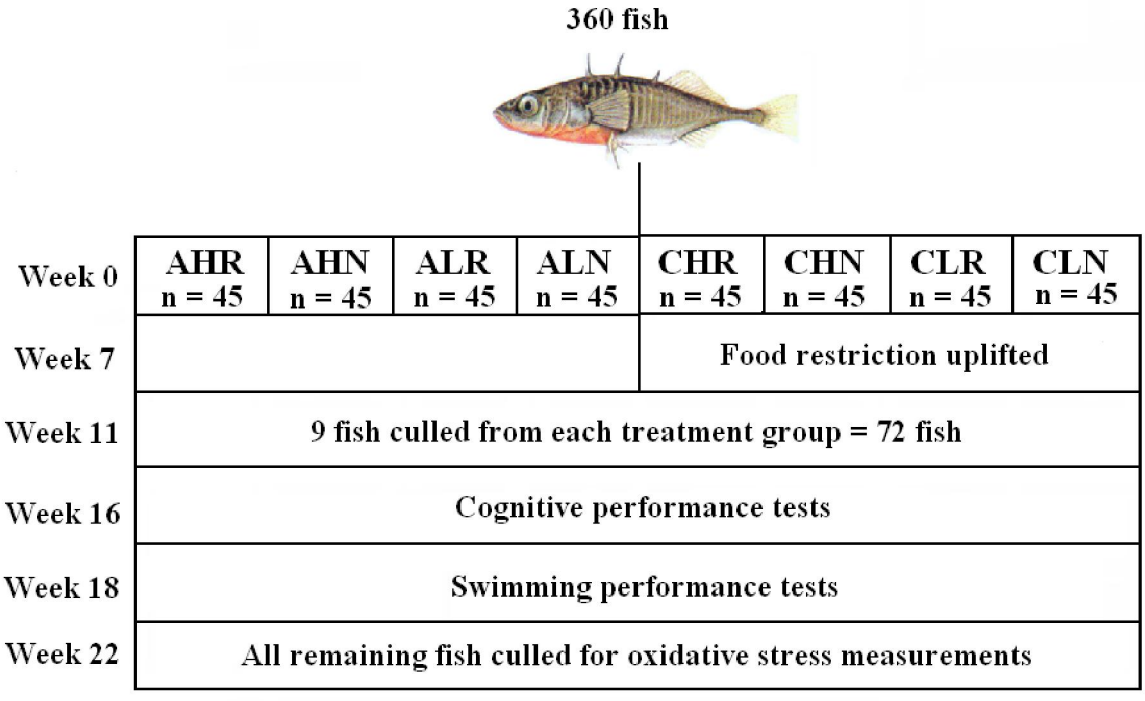


Figure 2.1 Schematic of experimental design. Fish were equally divided into the eight feeding treatments described in Table 2.1. At week 7 the food restriction was uplifted in the four compensatory growth regime fish (CHR, CHN, CLR and CLN). At week 11, nine fish were culled from each of the eight treatment groups. At week 16 and 18, all remaining fish underwent a cognitive performance assessment and swimming performance test, respectively. At week 22, all remaining fish were culled for oxidative stress measurements.

Appendix I gives a full description of the food preparation protocol. Most of the red colour of Chironomidae larvae can be attributed to the presence of a respiratory pigment of the haemoglobin type (Czeczuga 1961). However, the presence of a number of carotenoids including lutein has been previously identified (Czeczuga 1970). Therefore, Appendix II describes a high-performance liquid chromatography (HPLC) analysis carried out on the prepared food to ascertain whether any dietary antioxidants were present in the chironomid larvae used as the basic food prior to experimental supplementation that would confound the effects of the dietary supplements. This HPLC analysis also shows that the lutein was retained in the supplemented food after being thawed in water. This analysis was carried out in order to validate the food preparation protocol used in the present study.

2.3.4 Cognitive performance

The cognitive performance assessment consisted of 4240 trials, which were carried out on 120 mature sticklebacks in the week beginning the 18th October 2010 by quantifying performance in an active avoidance task. The sticklebacks were tested in the cognitive performance trials in a random order. A total of 14 fish were excluded from the analysis of cognitive performance in this task as after the acclimatisation period they continued to exhibit erratic and unsettled behaviour which would confound with the measurements used to measure cognitive performance. This time point was nine weeks after the restricted growth manipulation had been uplifted at the end of period 1, by which time the C growth regime fish had fully compensated in growth and converged in mean size with the A growth regime fish (see Figure 2.3).

An active avoidance paradigm was used to test the three-spined sticklebacks using a modified version of a shuttle box designed for the study of escape and avoidance in fish (Horner et al. 1961) (Figure 2.2). The shuttle box consisted of a plastic transparent tank (33 × 18 × 19 cm), which was subdivided into two well-distinguished compartments by a solid wedged-shaped hurdle. The bottom of the tank and the two longer sides of the tank were covered with black plastic making them impenetrable to light. The two shorter ends of the tank were transparent to permit light to pass through. The apparatus was filled with 50% fresh water and 50% of water taken from the home tank of the experimental fish. The water surface was 4cm above the top of the hurdle to provide an adequate margin for the fish to be able to pass between the two compartments. The water temperature was the same as that in the holding tanks of the experimental fish. Each fish was moved to the testing

tank, located in a quiet and dimmed experimental room adjacent to that containing the holding tanks, and left for ten minutes to acclimatise within the apparatus. The experiments were conducted blind, i.e. the observer did not know which treatment group each fish belonged to at the time of testing.

To begin the experiment, a red light (7.5W) illuminated the compartment the fish had settled in at a 10cm fixed distance, perpendicular to the transparent end of the tank. The red light (conditioned stimulus) was delivered as a means of warning the fish that an aversive stimulus would follow. In this experiment the aversive stimulus was a small net which was subsequently whirled to disturb the water in the compartment illuminated by the red light. In practice trials, all fish would actively avoid the swirling net and it was therefore deemed a suitable aversive stimulus.

The concept of the shuttle box test was to teach the sticklebacks to cross the hurdle to the opposite dark compartment within 15 seconds of the onset of the red light in order to avoid a swirling net. If the fish failed to do so and remained in the illuminated compartment for over 15 seconds, the swirling net would be delivered for a further 15 seconds along with the red light. Following each trial, the fish was allowed to rest for 30 seconds before the cycle was repeated again. A pass would be assigned to the trial if the fish succeeded in crossing the hurdle within 15 seconds of the red light. However, if the fish remained or returned to the illuminated compartment (which was illuminated for a total of 30 seconds in each trial) the trial would be scored as a fail. Escape latency was measured as the time it took the fish from the onset of the red light to cross the top of the hurdle into the opposite compartment in a successful trial. Each experimental fish participated in 40 consecutive trials that were each scored as either a pass or a fail. Therefore, it took a total of 50 minutes to test one fish including the ten minute acclimation period and the additional 30 second recuperation period between trials. Fish were observed by means of a mirror mounted above the tank so that the fish were not disturbed by the observer having to hover to view the fish from above. All 106 fish used in the cognitive performance experiment were then measured, weighed and returned to their original holding tank after the experiment.

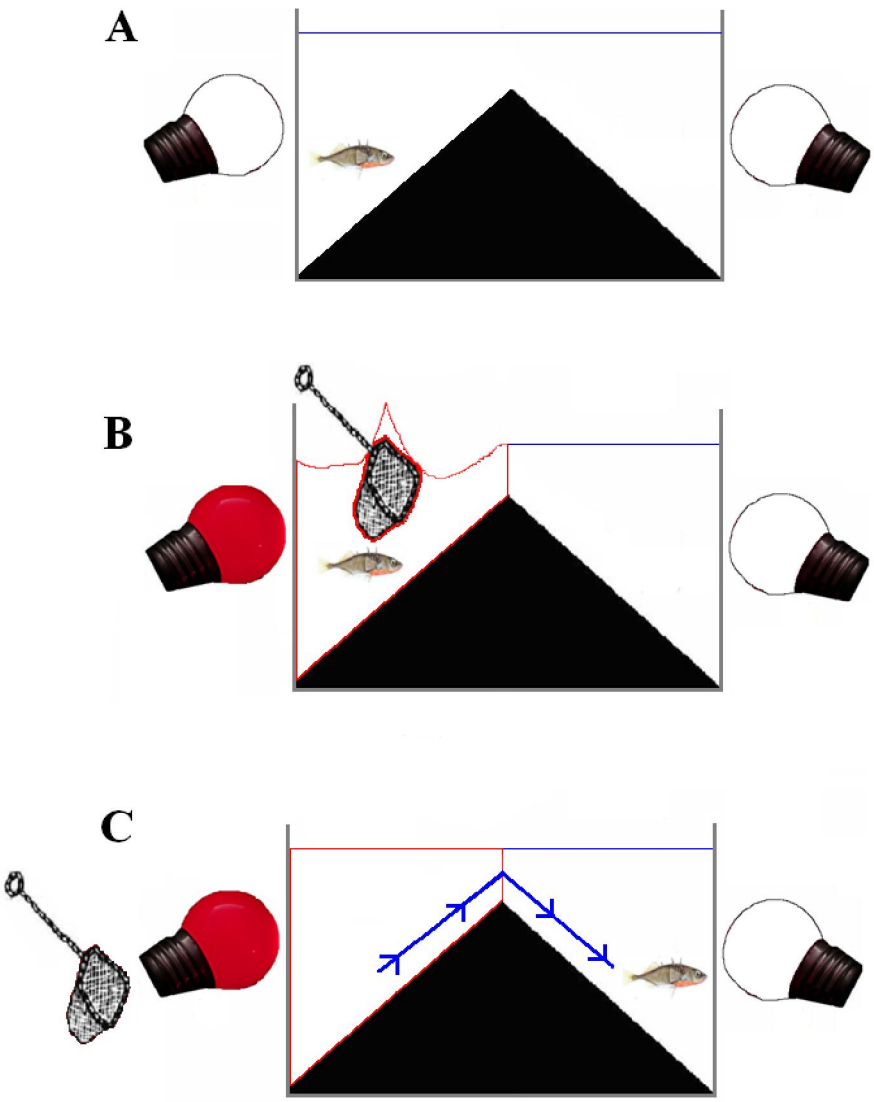


Figure 2.2 Schematic drawing of the shuttle box used for the active avoidance task. Scenario A depicts the situation at the start of each trial and during the recovery period between trials where no stimulus was present. Scenario B depicts a fail response from the fish, since it has failed to cross the tank within 15s of the conditioned stimulus (the red light becoming illuminated) and so is chased by the net (aversive stimulus). In scenario C (a pass response), the blue arrows indicate that the fish has crossed to the other side within 15s and so avoids the aversive stimulus of the net.

2.3.5 Swimming performance

Swimming performance was measured in the week beginning the 1st November 2010 and was carried out on the same 120 sticklebacks used to measure cognitive performance. This time point was after the fish had completed the active avoidance task. All 113 swimming trials were completed by the week beginning the 8th November 2010. The fish were tested in the swimming trials in the same order that they were tested in the active avoidance trials

in order for each fish to have a similar recovery period between the two performance tests. Swimming performance was measured in terms of swimming stamina, which was measured as the amount of time a fish could swim against a continuous strong current of water until fatigued. This experiment follows the experimental setup described in Álvarez & Metcalfe (2005), which has been previously used to measure swimming stamina in sticklebacks (Álvarez & Metcalfe 2005; Lee et al. 2010; Pike et al. 2010b). The swimming trials were conducted within the same room that the fish were housed in and the temperature matched the mean ambient conditions at the source river for that time of year. Therefore, the fish were not exposed to differences in water temperature between the swimming tunnel and their experimental holding tanks.

One at a time, the fish were placed in a cylindrical transparent tube within a swimming chamber (50 cm long, 20cm diameter) and exposed to a moderate water flow of 17.0 cm s^{-1} for five minutes. This allowed the fish to acclimatise to the apparatus and also orientate themselves by swimming against the inflow of water. After this acclimatization period, the water flow rate was increased to 34.9 cm s^{-1}. These low and high water flow rates were selected based on previous work carried out by Lee et al. (2010), who used the same swimming chamber apparatus for measuring sustained swimming performance in sticklebacks from the same source population. The high flow rate in the current experiment is comparable to the maximum velocity that the sticklebacks in Lee et al. (2010) were able to sustain (Lee et al. 2010).

The time taken until the fish was fatigued at the high flow rate was recorded as a measurement of swimming stamina. Fatigue was defined as when a fish was no longer able to swim against the inflow of water and was pushed back downstream against a fine mesh grid at the end of the cylinder for more than 5 seconds. At this point, the water flow was immediately turned off and the fish left to recover for two minutes within the swimming chamber before being measured, weighed and returned to its original holding tank. All fish quickly recovered from the trials and were swimming around normally in under 5 minutes. A total of 7 fish were excluded from the analysis of swimming performance in this task. These fish were observed using their tail fin to support themselves against the fine mesh grid at the end of the cylinder and the onward facing water current, so that it was not possible to measure their true swimming stamina.

2.3.6 Oxidative stress assays

All remaining fish were culled, weighed and measured for their final growth measurements on the 29th November 2010. The fish were then frozen in the dark at -80°C until June 2011, when they were analysed for oxidative stress status. However, alongside the 72 fish culled for oxidative stress measurements on 20th September, some of these culled fish were also lost in the freezer accident. Therefore, the oxidative stress analyses were carried out on the remaining 80 fish following the same methods that are fully described in Chapter 3.

2.3.7 Statistical analysis of growth

The fish were measured at the beginning of the experiment before growth manipulation commenced, twice during the growth manipulation period (Period 1) and five times after the restricted ration had been uplifted (Period 2). In order to prevent pseudoreplication, the mean standard body length and mass of all fish from each original tank of 15 fish, at each sampling point was used as the data point to analyse growth. Once the fish had been transferred to smaller tanks, their growth continued to be analysed in respect to the tank of 15 fish of which they originally came from. This was also necessary due to being unable to identify and track the growth trajectory of each individual fish in their initial large tanks prior to being re-housed into smaller tanks. Some mortality occurred during the experiment and tank means were based on surviving fish only in the analysis of growth. A total of 250 fish were used in this growth analysis up until the 20th September 2010 (117 C growth regime fish and 133 A growth regime fish). On the 20th September 2010, three fish from each of the 24 tanks were culled for oxidative stress measurements and were therefore missing from subsequent growth analyses after this time point. Differences in standard body length and mass between the *ad libitum* and compensatory growth regimes were tested using a multivariate analysis of variance (MANOVA), as were differences in standard body length and mass between all eight feeding treatments.

2.3.8 Statistical analysis of cognitive performance

The effects of manipulating both growth regime and diet on active avoidance response and escape latency were analysed using a generalised linear mixed model (GLZMM) fitted by the Laplace approximation and a general linear mixed model (GLMM) respectively, with growth regime (*ad libitum* fed or compensatory growth, denoted A or C, respectively),

carotenoid supplementation (high or low, denoted H or L, respectively), resveratrol supplementation (present or absent, denoted R for present and N for not present in the diet) and trial number included as fixed effects. Body length and mass at time of cognitive testing were included as covariates. Although there were no mean size differences at this time point between growth regimes or feeding treatments, by including these body measurements this would account for between-individual size differences. Fish identification number was included as a random factor to control for the 40 repeated trials carried out on each fish. Fish identification number was nested within tank number which was also included as a random factor to control for tank effects, plus all two-way interactions among variables. The active avoidance response was measured as a binary response (pass or fail). The distribution of the continuous latency data was normal and therefore did not require transformation prior to being analysed. Non-significant variables were sequentially dropped from the analyses so that the final models only included significant terms apart from main effects that occurred in significant two-way interactions.

2.3.9 Statistical analysis of swimming performance

The effect of both growth regime and dietary manipulation on swimming stamina was analysed using a GLMM, with growth regime (A or C), carotenoid supplementation (H or L), and resveratrol supplementation (R or N) included as fixed effects. Body length and mass at time of testing were included as covariates, as body size is known to correlate with swimming performance in fish and therefore this would account for between-individual differences in size (Videler 1993). Tank number was included as a random factor to control for tank effects, plus all two-way interactions among variables. Swimming performance data were positively skewed and were therefore log-transformed to meet the assumptions of parametric analysis. Non-significant variables were sequentially dropped from the analysis so that the final model only included significant terms.

2.3.10 Statistical analysis of oxidative stress

The effect of growth regime and dietary manipulation on each of the four measurements used to assess oxidative stress status, (superoxide dismutase (SOD) activity, glutathione peroxidase (GPx) activity, total protein content and protein carbonyl content) was analysed separately using GLMMs, with growth regime (A or C), carotenoid supplementation (H or L), and resveratrol supplementation (R or N) as fixed effects, final body length and mass as

covariates and tank number as a random factor, plus all two-way interactions among variables. SOD, GPx and protein carbonyl content data were all positively skewed and were therefore log, sqrt and sqrt transformed, respectively before statistical analysis.

All means are described with standard errors and all analyses in the present chapter were carried out using R (R Core Development Team, version 2.15.0). The function lmer within the lme4 package was used to fit the GLZMM for active avoidance response in the cognitive performance task. The function lme within the nlme package was used to fit the GLMMs for escape latency, swimming endurance and all measurements of oxidative stress. Significant results were defined as $p < 0.05$. Non-significant variables were sequentially dropped from each analysis so that the final models only included significant terms apart from main effects that occurred in significant two-way interactions.

2.4 RESULTS

2.4.1 Compensatory growth response after food restriction

At the start of the experiment there were no differences in either the mean standard length or mass between the two growth regime groups (A and C) (MANOVA: Wilk's $\lambda = 0.855$, $F_{2,21} = 1.783$, $p = 0.193$) (Figure 2.3), or among the eight feeding treatments overall (see Table 2.1 in methods for description of treatment groups) (MANOVA: Wilk's $\lambda = 0.372$, $F_{14,30} = 1.370$, $p = 0.227$). However, after the 7-week manipulation of growth regimes (end of Period 1) there were significant differences in both mean standard length and mass between the two growth regime groups (A and C) (MANOVA: Wilk's $\lambda = 0.161$, $F_{2,21} = 54.655$, $p < 0.001$). At this time point, the C fish were 4.1% shorter in mean standard length (ANOVA, $F_{1,22} = 7.74$, $p = 0.011$) and 23.7% lighter in mean mass (ANOVA, $F_{1,22} = 33.840$, $p<0.001$) than A fish.

While there was a significant difference in mean standard length and mass at the end of Period 1 among the eight feeding treatments overall (MANOVA: Wilk's $\lambda= 0.081$, $F_{14,30} = 5.378$, $p < 0.001$), this was really just a consequence of the difference between the two growth regime groups, since within each of these there were no effects of resveratrol supplementation or carotenoid supplementation (MANOVA comparing diet groups within the A growth regime: resveratrol: Wilk's $\lambda= 0.876$, $F_{2,9} = 0.638$, $p = 0.551$; carotenoid: Wilk's $\lambda= 0.675$, $F_{2,9} = 2.160$, $p = 0.171$; comparison within the C growth regime:

resveratrol: Wilk's $\lambda = 0.858$, $F_{2,9} = 0.7469$, $p = 0.501$; carotenoid: Wilk's $\lambda = 0.816$, $F_{2,9} = 1.018$, $p = 0.400$). The differences in size began to disappear once the C growth regime fish were transferred onto the A growth regime diet. After 9 weeks of uplifting the C growth regime, full compensation had occurred as there were no longer significant differences in size between the two growth regime groups (A and C) (MANOVA: Wilk's $\lambda = 0.772$, $F_{2,20} = 2.947$, $p = 0.076$) (Figure 2.3) or among the eight feeding treatments overall (MANOVA: Wilk's $\lambda = 0.325$, $F_{14,28} = 1.508$, $p = 0.172$).

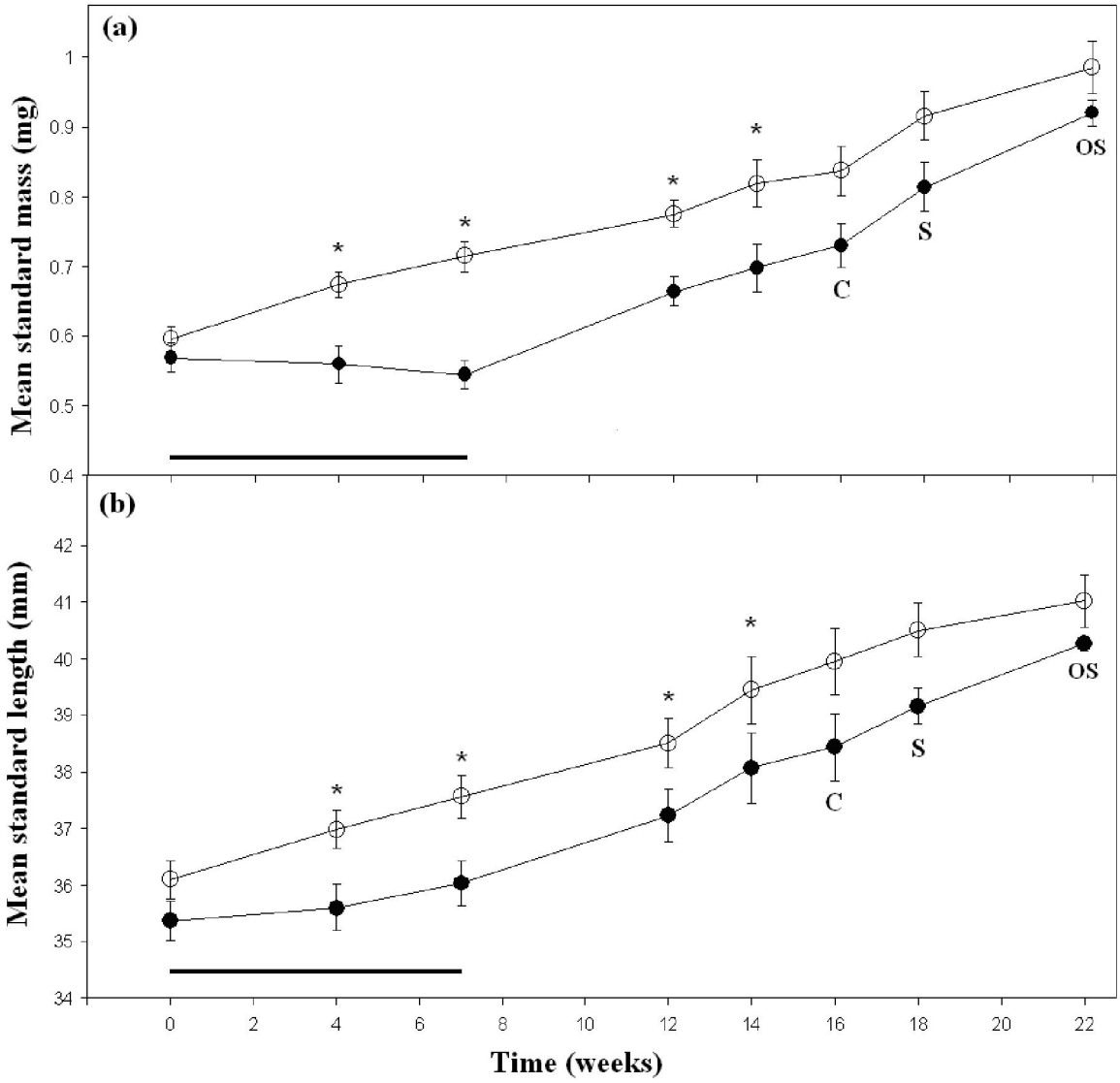


Figure 2.3 Growth trajectories for mean ± s.e. (a) mass and (b) standard length of three-spined sticklebacks in relation to growth regime (*ad libitum*, open circles; compensatory growth, closed circles). The thick horizontal line indicates the period of growth manipulation (7 weeks), after which point all fish were fed *ad libitum* but were still maintained on their original dietary supplementations. Asterisks indicate significant differences in mass or length between the A and C growth regime groups ($p<0.05$). "C" and "S" indicate the timing of the cognitive and swimming performance trials, respectively, after full compensation had been achieved in the C growth regime group. "OS" indicates when the experiment concluded and the fish were culled for their oxidative stress measurements.

2.4.2 Active avoidance response in cognitive performance test

A non-linear, saturating relationship was found between trial number and the percentage of fish (over all treatments) that passed the test, indicating that the more experience the fish had of the active avoidance test the better they performed (Table 2.2 and Figure 2.4). The total percentage of fish that passed significantly increased with trial number regardless of their treatment group (GLZMM, z = 8.469, $p < 0.001$). This suggests operant learning had taken place and that the fish were capable of learning to actively avoid the aversive stimulus. A significant difference in active avoidance response was found between the A growth regime fish and those that had experienced the compensatory growth regime (GLMM, $z = 1.959$, $p = 0.05$), with A growth regime fish performing significantly better in terms of the total percentage of fish that passed each of the 40 trials (Table 2.2 and Figure 2.4). A significant interaction was found between trial number and whether fish were fed a diet supplemented with resveratrol or not (GLZMM, $z = -2.822$, $p = 0.005$). Fish receiving resveratrol had a higher initial success rate in passing the task, in comparison with the fish fed a diet lacking in resveratrol. Therefore fish fed a diet supplemented with resveratrol learnt the task quicker than fish that were not fed resveratrol (Table 2.2 and Figure 2.5). However, their subsequent rate of improvement was lower, leading to the two groups having similar success rates by the end of the experiment (Figure 2.5). Therefore, there was no net benefit of resveratrol overall. There was no difference between fish fed a diet high or low in carotenoids in terms of their active avoidance response success (GLZMM, $z = 0.907$, $p = 0.364$). Although there were no longer differences between treatment groups in terms of body size (Figure 2.3), there were positive effects of individual differences in body length and body mass at time of testing (i.e. larger fish performed better in the task; Table 2.2).

Table 2.2 Results of a generalised linear mixed model examining active avoidance response in relation to trial number, growth regime, carotenoid supplementation and resveratrol supplementation. Body length and mass were included as covariates. Fish ID was nested within tank ID which were both included as random factors. Non-significant variables were sequentially dropped from the analysis apart from main effects occurring in significant two-way interactions. The data are binary (either pass or fail) for the active avoidance response.

Final model	Estimate	SE	*z*	*p*
Trial	0.038	0.004	8.469	<0.001
Growth regime (A)	0.423	0.216	1.959	0.050
Length	0.186	0.070	2.652	0.008
Mass	3.319	1.216	2.729	0.006
Resveratrol (Y)	0.263	0.250	1.054	0.292
Resveratrol (Y) × Trial	-0.018	0.006	-2.822	0.005
Carotenoid (H)	0.191	0.210	0.907	0.364

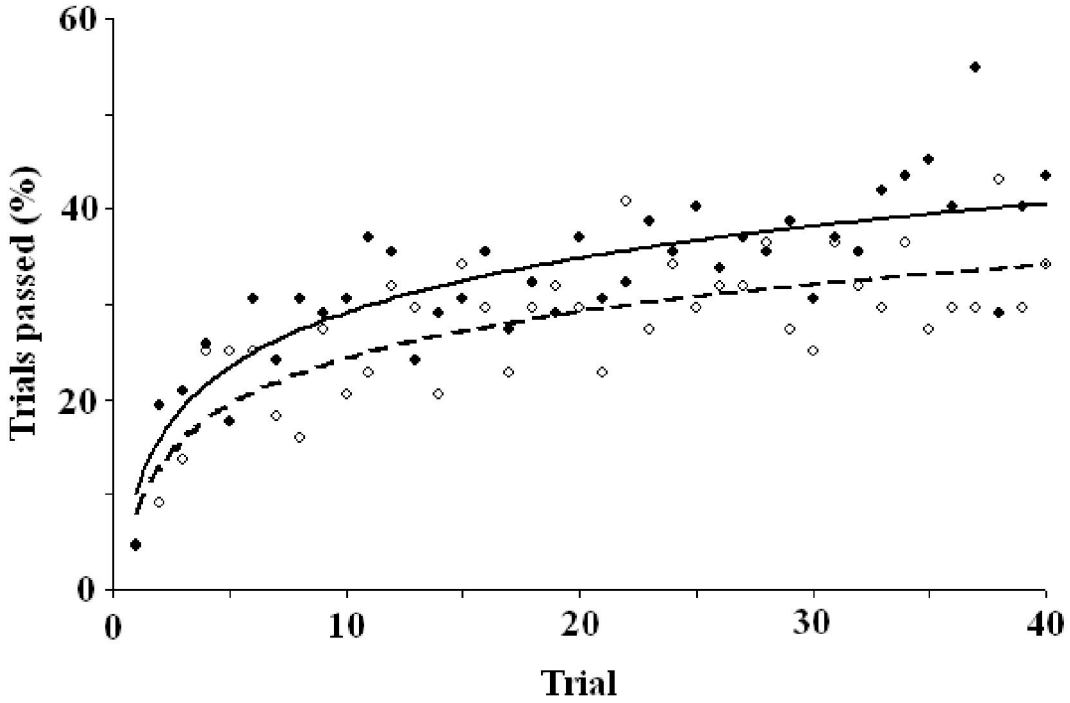


Figure 2.4 Percentage of successful trials for the two growth regime groups (A and C) at each of the 40 trial points; n = 106 fish. Values are plotted separately for the two growth regimes (A group- solid circles and solid logarithmic regression line; C group-open circles and dashed logarithmic regression line).

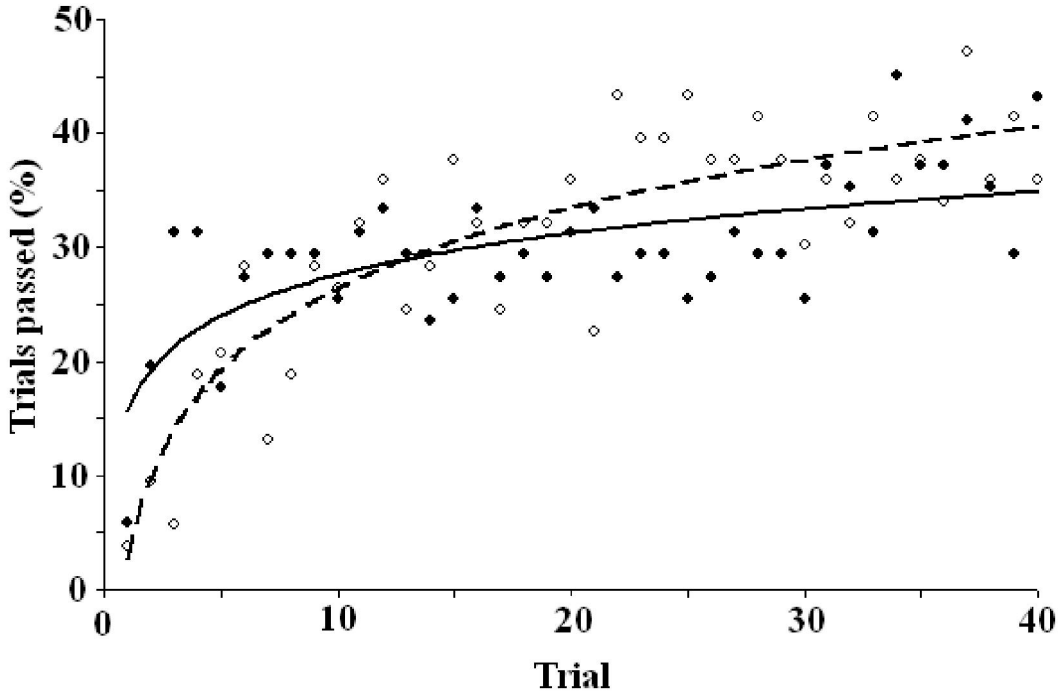


Figure 2.5 Percentage of successful trials for fish fed either a diet supplemented with resveratrol (solid circles and solid logarithmic regression line) or a diet lacking in resveratrol (open circles and dashed logarithmic regression line) at each of the 40 trial points; n = 106 fish.

2.4.3 Escape latency in cognitive performance test

A negative relationship was found between trial number and escape latency (Figure 2.6). The mean escape latency for all fish significantly decreased with trial number regardless of what treatment group they belonged to (GLMM, t_{1197} = -3.358, p<0.001), indicating that the more experience the fish had of the active avoidance test, the faster they escaped to the safe half of the tank after the onset of the conditioned stimulus (red light). There were no significant differences in escape latency between the control growth and compensatory growth regime fish (GLMM, t_{1197} = 1.388, p = 0.165). However, there was a significant effect of resveratrol (GLMM, t_{1197} = -7.445, p < 0.001), with fish fed resveratrol escaping significantly faster in the 40 trials overall (See Table 2.3 and Figure 2.7). A significant interaction was found between growth regime and whether fish were fed a diet supplemented with a high or low dose of carotenoids (GLMM, t_{1197} = -2.593, p = 0.010). For fish on the *ad libitum* growth regime, a significantly faster escape response was found in fish fed a diet high in carotenoids in comparison with fish fed a diet low in carotenoids (See Table 2.3 and Figure 2.8). However, this pattern was absent in the compensatory growth regime fish. A significant interaction was also found between trial number and

whether fish were fed a diet supplemented with a high or low dose of carotenoids (GLMM, $t_{1197} = 2.090$, $p = 0.037$).

Table 2.3 Results of a general linear mixed model examining escape latency in relation to trial number, growth regime, carotenoid supplementation, resveratrol supplementation. Body length and mass were included as covariates. Fish ID was nested within tank ID which were both included as random factors. Non-significant variables were sequentially dropped from the analysis apart from main effects occurring in significant two-way interactions.

Final model	Estimate	SE	DF	*t*	*p*
Trial	-0.073	0.022	1197	-3.358	<0.001
Regime (A)	0.686	0.494	1197	1.388	0.165
Resveratrol (Y)	-1.466	0.198	1197	-7.445	<0.001
Carotenoid (H)	-0.535	0.467	1197	-1.147	0.252
Carotenoid (H) ×Regime (A)	-1.961	0.756	1197	-2.593	0.010
Carotenoid (H) ×Trial	0.069	0.033	1197	2.090	0.037

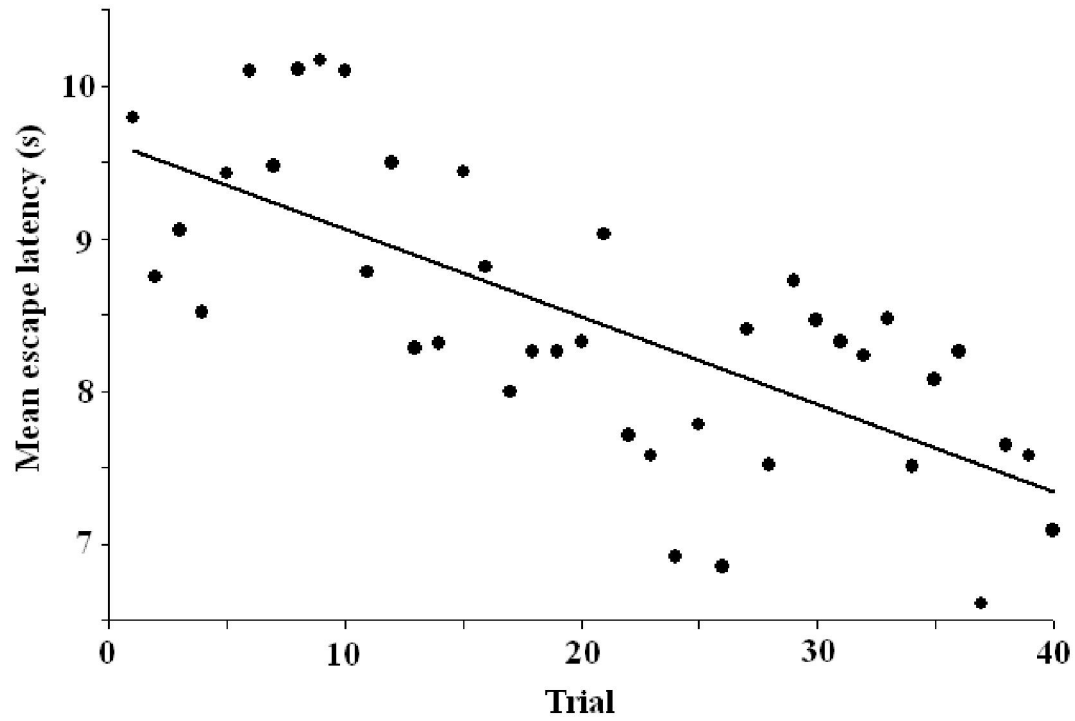


Figure 2.6 Mean escape latency in seconds for all fish fitted across the 40 trials in the active avoidance task. The line indicates the significant linear regression of escape latency on trial number (see Table 2.3); n = 106 fish.

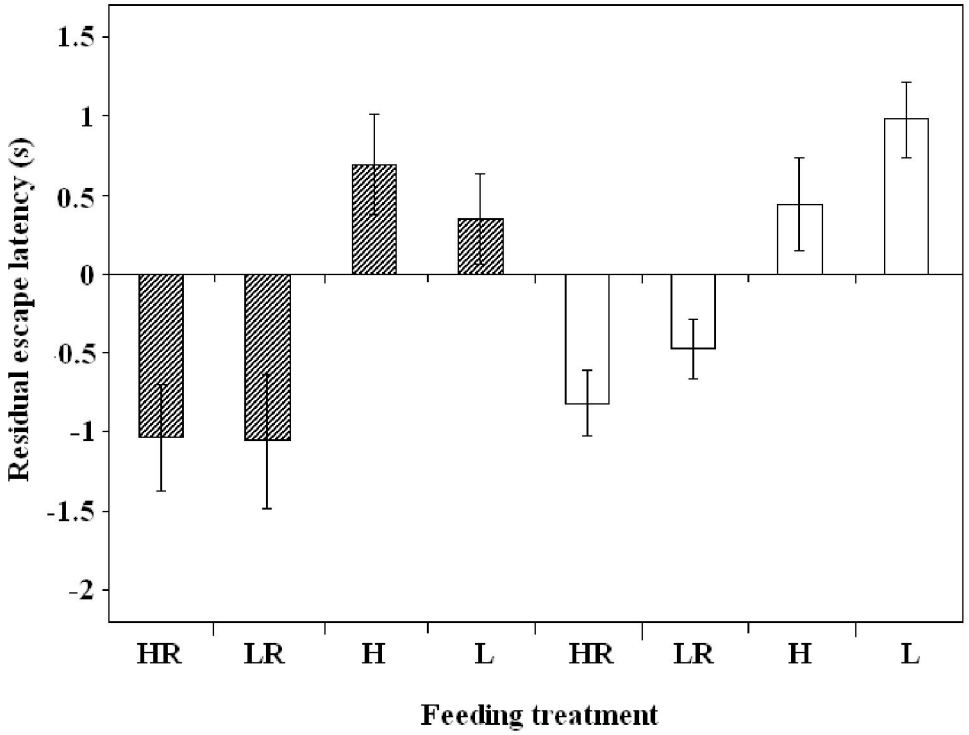


Figure 2.7 Mean ± s.e. residual escape latency in seconds over all 40 trials, after taking account of the effect of trial number. Data are expressed as least square means, and are plotted separately for each of the 8 treatment groups. Within each of the two growth regimes (A - open bars and C - hatched bars) the high carotenoid + resveratrol, low carotenoid + resveratrol, high carotenoid no resveratrol and low carotenoid no resveratrol treatments are denoted HR, LR, HN and LN, respectively; n = 106 fish.

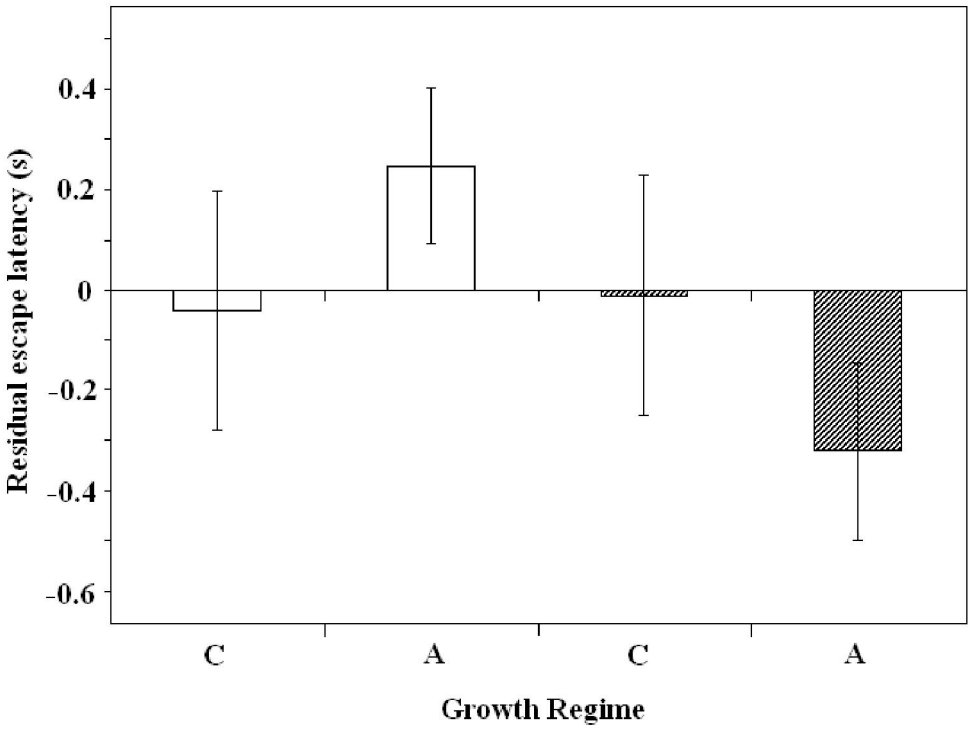


Figure 2.8 Mean ± s.e. residual escape latency in seconds over all 40 trials after taking account of the effect of trial number. Data are plotted separately for fish fed under a compensatory growth regime (C) or an *ad libitum* growth regime (A), and fed either a diet high in carotenoids (hatched bars) or low in carotenoids (open bars); n = 106 fish.

2.4.4 Swimming performance

The fish that experienced compensatory growth had significantly poorer swimming stamina than *ad libitum* growth regime fish (GLMM, $t_{53} = 2.392$, $p = 0.020$) (Figure 2.9, Table 2.4). Although there were no longer differences between treatment groups in terms of body size (Figure 2.3), there were positive effects of individual differences at the time of testing in body length but not body mass (i.e. longer fish had greater swimming stamina; Table 2.4) (GLMM, $t_{57} = 2.229$, $p = 0.029$). However, there were no differences in swimming performance between fish fed a diet high or low in carotenoids, or between fish fed a diet with resveratrol or not (Table 2.4, Figure 2.9). Figure 2.9 shows that the mean stamina for the HR group had a markedly larger error bar in comparison with the other treatment groups; this was not due to a smaller sample size (n = 16 fish for that group, which is higher than the average), but to two fish exhibiting exceptionally high swimming stamina's of 5100 and 5440 seconds compared with the other fish within the HR group (which had a mean swimming stamina of 165 seconds). The results of the swimming experiment were not affected when these two outliers were excluded from the data set.

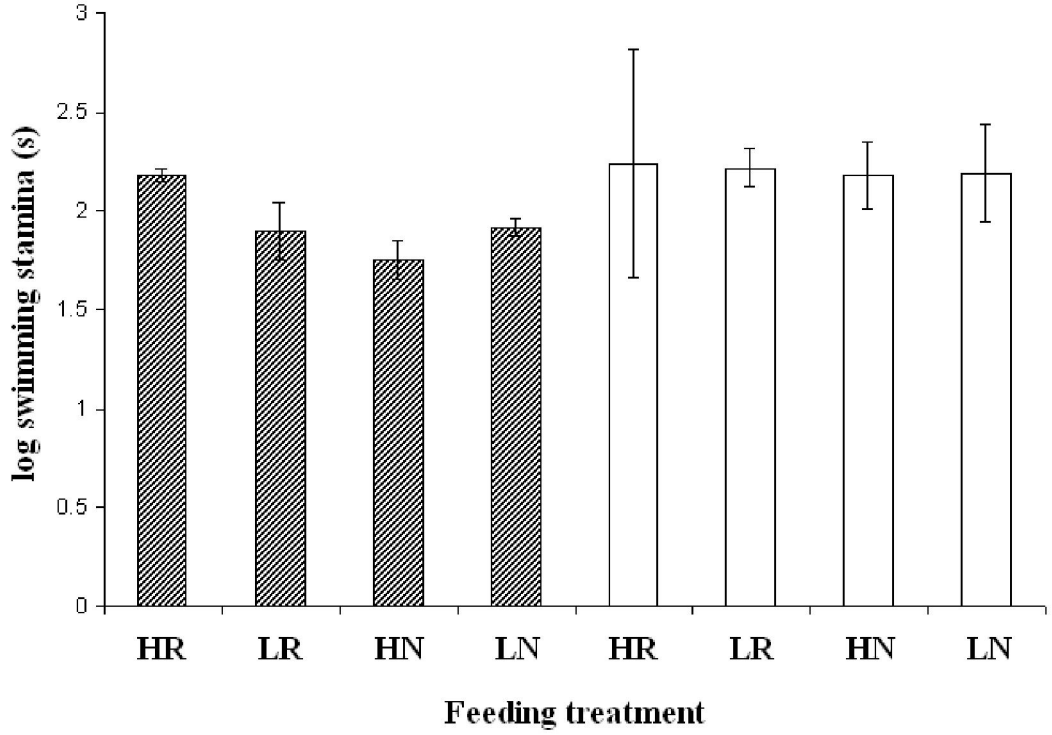


Figure 2.9 Mean ± s.e. swimming stamina (log transformed) in relation to growth regime (A growth regime - open bars and C growth regime - hatched bars). Data are expressed as least square means and standard body length and mass were included as covariates to control for individual differences at the time of testing. High carotenoid + resveratrol, low carotenoid + resveratrol, high carotenoid no resveratrol and low carotenoid no resveratrol denoted by HR, LR, HN and LN, respectively; n = 113 fish.

Table 2.4 Results of a general linear mixed model examining swimming stamina in relation to growth regime, carotenoid and resveratrol supplementation. Standard body length and mass at time of testing was included as a covariate. Tank was included as a random factor. Non-significant variables were sequentially dropped from the analysis.

Final model	SE	DF	*t*	*p*
Growth regime (A)	0.244	53	2.392	0.020
Carotenoid (H)	0.248	51	0.166	0.869
Resveratrol (Y)	0.242	52	0.951	0.346
Length	0.029	57	2.229	0.029

2.4.5 Oxidative stress

The fish that experienced compensatory growth (the C treatment group) had significantly lower SOD activity than A growth fish (GLMM, t_{39} = -2.065, p = 0.046) (Figure 2.10, Table 2.5), while those that had been fed resveratrol had significantly lower SOD activity than those that had not (GLMM, t_{39} = -2.176, p = 0.036) (Figure 2.10, Table 2.5). There was no difference in GPx activity or protein carbonyl content in relation to growth regime or dietary supplementation (Figure 2.11 and Figure 2.12).

Table 2.5 Results of a general linear mixed model examining superoxide dismutase content (SOD) in relation to growth regime, carotenoid and resveratrol supplementation. Final body length and mass were included as covariates. Tank was included as a random factor. Non-significant variables were sequentially dropped from the analysis.

Final model	SE	DF	*t*	*p*
Growth regime (C)	0.136	39	-2.065	0.046
Resveratrol (Y)	0.131	39	-2.176	0.036
Carotenoid (L)	0.135	38	-0.737	0.466

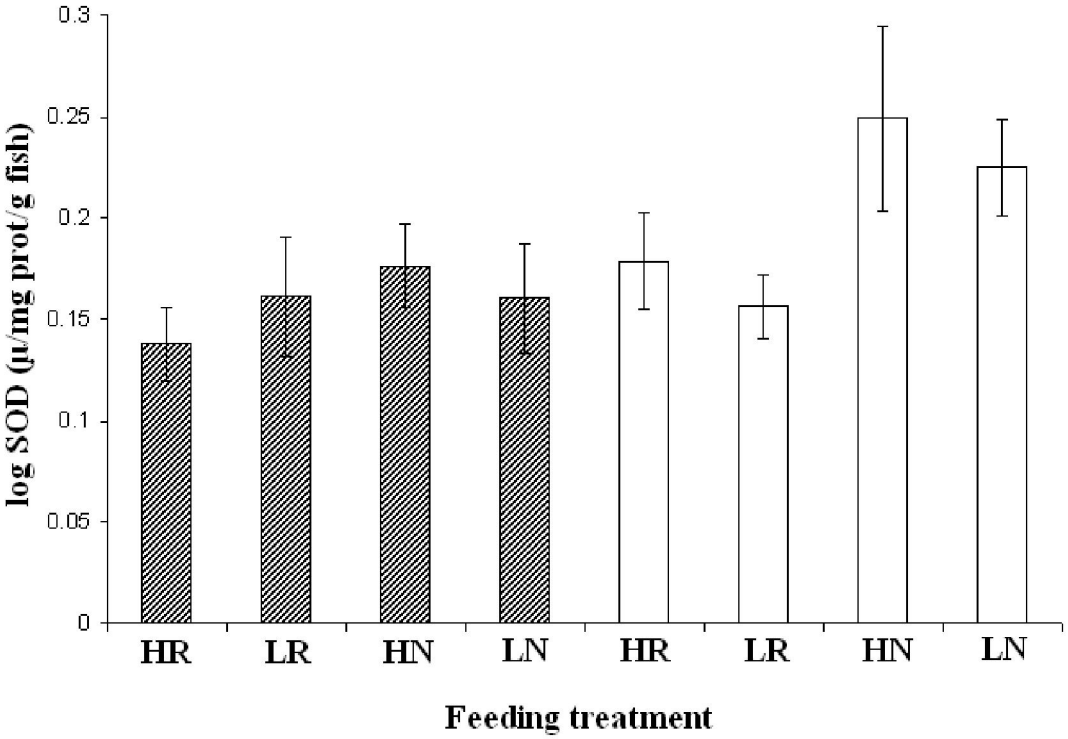


Figure 2.10 Mean ± s.e. superoxide dismutase content (log transformed) in relation to feeding treatment group (A growth regime - open bars and C growth regime - hatched bars). High carotenoid + resveratrol, low carotenoid + resveratrol, high carotenoid no resveratrol and low carotenoid no resveratrol denoted by HR, LR, HN and LN, respectively; n = 80 fish.

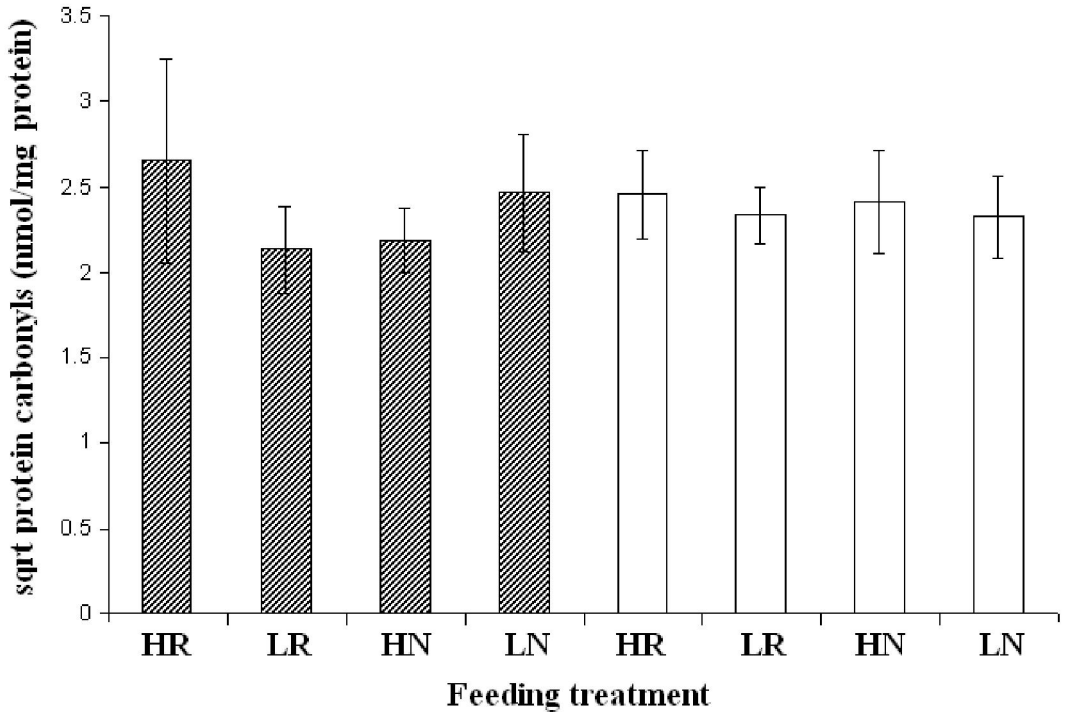


Figure 2.11 Mean ± s.e. protein carbonyl content (sqrt transformed) in relation to feeding treatment group (A growth regime - open bars and C growth regime - hatched bars). High carotenoid + resveratrol, low carotenoid + resveratrol, high carotenoid no resveratrol and low carotenoid no resveratrol denoted by HR, LR, HN and LN, respectively; n = 80 fish.

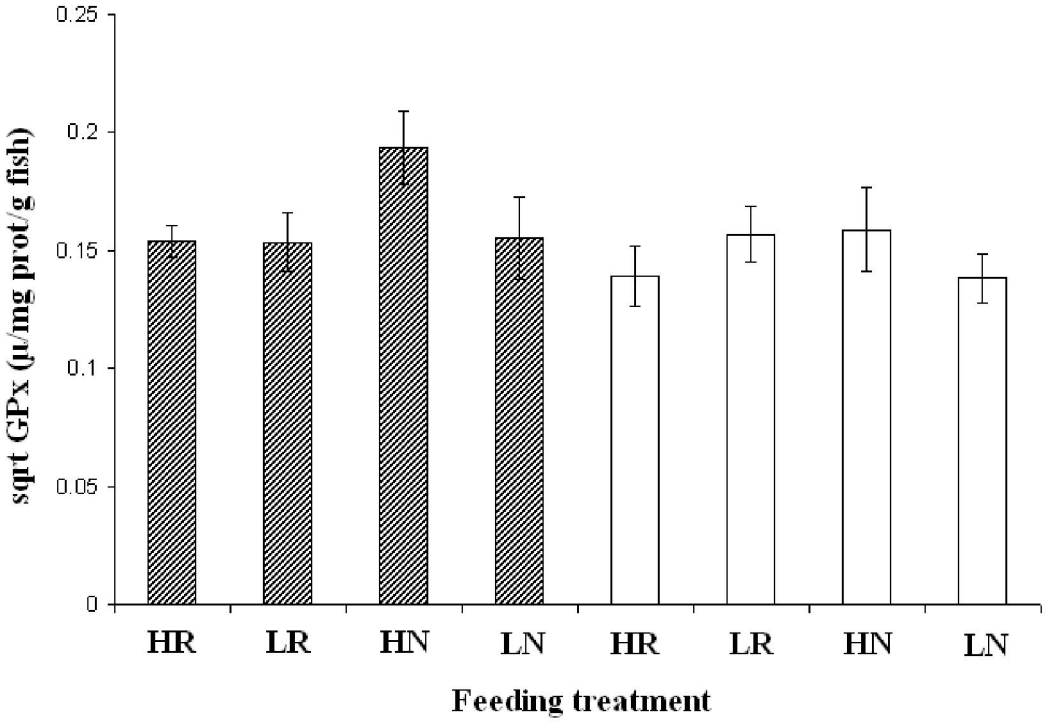


Figure 2.12 Mean ± s.e. glutathione peroxidase content (sqrt transformed) in relation to feeding treatment group (A growth regime - open bars and C growth regime - hashed bars). High carotenoid + resveratrol, low carotenoid + resveratrol, high carotenoid no resveratrol and low carotenoid no resveratrol denoted by HR, LR, HN and LN, respectively; n = 80 fish.

2.5 DISCUSSION

2.5.1 Compensatory growth response

In accordance with previous work on sticklebacks (Zhu et al. 2003; Inness & Metcalfe 2008; Lee et al. 2010), this study found that dietary restriction in early life resulted in a compensatory growth response after the food restriction had been uplifted. A 7-week period of food restriction had effects on the growth trajectory of the fish during the food restriction period itself but also on the subsequent growth trajectories up until nine weeks after this food restriction had been uplifted. This deviance in growth trajectories during the food restriction was buffered by the subsequent compensation period in terms of both length and mass. Therefore, the compensatory growth regime fully compensated in terms of both energy reserves and skeletal growth.

In the present study, the C growth regime fish were 4.1% shorter and weighed 23.7% less than the A growth regime fish at the end of the period of restriction. Although these were significant differences in terms of both length and mass between the A and C growth

regime fish, the differences were not as profound as those found in a previous study (where the same regime resulted in restricted fish being 5.3% shorter and 35.5% lighter than the fish fed *ad libitum* after just 21 days; (Álvarez & Metcalfe 2005)). While the initial size of the fish was comparable in the two experiments, the ambient temperatures were slightly different, which may explain this growth difference as temperature is an environmental factor which is known to influence early growth (Lee et al. 2010). The smaller effect of the manipulation on size at the end of the restriction period may explain the relatively modest compensatory response, with no sign of marked acceleration of growth once the restriction was lifted. However, the regime did have significant effects on later performance.

2.5.2 Cognitive performance

Compensatory growth was found to result in significant costs in later-life cognitive performance. Fish in the A growth regime performed significantly better than those in the C growth regime in terms of cognitive performance overall in the 40 trials, being more likely to succeed in the task at every stage of training. This result suggests that the limiting resources available in early life in the C growth regime group may have resulted in a physiological trade-off between growth and somatic maintenance, possibly in terms of neuroprotection. Regardless of which, compensatory growth negatively affected later-life cognitive performance and therefore suggests a trade-off between growth and cognitive performance.

In this experiment, resveratrol-fed fish performed the task more successfully in earlier trials than fish not receiving resveratrol, although by the 13th trial there were no longer differences in performance and even a slight trend for the non-resveratrol fish to do better. Resveratrol-fed fish may have habituated to the task earlier and therefore their performance may have possibly reached a plateau for this reason. Perhaps the resveratrol-fed fish were bolder and had become more tolerant of the aversive stimulus. In hindsight it would have been interesting to have carried out the trials for slightly longer in order to determine whether the performance of fish not fed resveratrol would have also reached a plateau soon after the 40 trials or continued to improve. Another possible improvement to the shuttle box trials carried out in the present study would be to implement a stronger aversive stimulus to avoid the fish habituating in the trials. For example, mild electric shocks have been successfully implemented as an aversive stimulus in several studies with laboratory

mice (Clark et al. 2003), goldfish *Carassius auratus* and zebrafish *Danio rerio* (Xu & Goetz 2012).

Even though the fish fed a diet with resveratrol did not perform better in the task overall, the fact that they were faster learners is of biological relevance. For example, there would be clear fitness benefits if the aversive stimulus had been a living predator as opposed to the swirling net used in this experiment. Having a quick response to the aversive environmental cues in this cognitive performance test suggests dietary antioxidants such as resveratrol could play a key role in how quickly fish could respond to approaching predators, therefore increasing their chance in escaping death. Considering predation is suggested to be one of the strongest agents of selection, this result is extremely important in a real world context as these results suggest that nutrients such as resveratrol can improve escape response which has clear potential fitness consequences. In this case, it is not relevant that the fish fed a diet lacking in resveratrol eventually performed just as well as the fish fed resveratrol, as in a real-life scenario they may have already been killed by a predator by failing to actively avoid the predator early enough. It should be noted that resveratrol is not present naturally in the diet of three-spined sticklebacks but has been used in the present study to demonstrate the importance of key nutrients such as dietary antioxidants in mediating life history trade-offs.

It has been previously shown that resveratrol retards the age-related decline in cognitive performance in another short-lived fish *Nothobranchius furzeri,* where old fish fed a diet supplemented in resveratrol performed better than fish fed a diet lacking in resveratrol (Valenzano et al. 2006b). However, the experiment only measured success over ten consecutive trials in comparison with the present study, which measured success over 40 consecutive trials to obtain a more detailed and comprehensive result. For instance, although resveratrol-fed fish learnt the task quicker within the first ten trials, by continuing the experiment in the present study with a further 30 trials it became transparent that the fish not fed resveratrol were able to achieve a comparable pass score soon after the 10^{th} trial. A more recent study by Yu et al. (2012) used a protocol in which fish were tested over 50 trials using the same cognitive task used in the present study. Although Yu et al. (2012) found resveratrol-fed fishes performed significantly better over the 50 trials than fish not fed resveratrol, the range in the mean frequency of correct avoidance responses was more comparable to the results in the present study rather than the exceptional results produced in Valenzano et al. (2006).

There was no apparent difference in the cognitive performance of fish fed low versus high doses of carotenoids, suggesting that carotenoids did not play a role in mediating any trade-off between growth and later-life cognitive performance. Carotenoids may not have important roles as antioxidants in the body when additional antioxidants such as resveratrol are present in the diet. It has been previously noted that the function of carotenoids as antioxidants is often debatable and contingent on the presence of other antioxidants such as polyphenolic compounds (Catoni et al. 2008). However, even though carotenoids in the present study were not found to have a role in influencing cognitive performance, they have been found to influence life history traits such as reproduction and immunity (Chew & Park 2004). Therefore, this suggests carotenoids may be important in mediating trade-offs between growth and reproductive performance for example - a topic that is investigated in Chapters 3, 4 and 5.

The results of the present study demonstrate that food supplemented with resveratrol had a significant beneficial effect on cognitive performance in comparison with same-age controls. However this significant effect is considerably weaker in comparison with the results found in the Valenzano et al. (2006) study. The exceptional results of resveratrol in Valenzano et al. (2006) stand out as a contradiction to the increasing trend in the literature which suggests a lack of resveratrol benefits in higher-order animals (Hector et al. 2012). *Nothobranchius furzeri*, the species used by Valenzano et al. (2006), is often selected in ageing research because of its conveniently short lifespan (Terzibasi et al. 2007). It is possible that resveratrol may be acting on the naturally evolved mechanism that causes its condensed lifespan. The species is known to have higher incidences of age-dependent liver and kidney problems which may be a potential target for resveratrol (Di Cicco et al. 2011). This may explain the exceptional differences in lifespan and performance between fish fed resveratrol or not seen in Valenzano et al. (2006). Alternatively, perhaps the striking results of Valenzano et al. (2006) could be attributed to the fish being tested at a more senescent state in comparison with the sticklebacks in the present study. For example, in Valenzano et al. (2006), *Nothobranchius furzeri* were tested at nine weeks old, near the end of their lives (their maximum recorded lifespan is only 13 weeks in captivity).

The positive effect of resveratrol on cognitive performance in the present study could be of relevance to studies investigating the prevention of age-related diseases in humans. The results provide circumstantial support for the hypothesis that the anti-ageing properties of resveratrol could be secondary to its neuroprotective actions, which are well reviewed in

Robb & Stuart (2010). For example, resveratrol has been found to have a protective effect on lipopolysaccharide-induced oxidative stress in rat brain (Sebai et al. 2009). Resveratrol has also been found to protect against oxidative stress-induced neuronal death in cultured cells *in vitro* (Fukui et al. 2010).

2.5.3 Swimming performance

It has previously been shown that compensatory growth prompted by either a nutritional deficit in early life or through manipulations in environmental temperature can subsequently affect later-life swimming performance in three-spined sticklebacks (Álvarez & Metcalfe 2005; Lee et al. 2010). The results of the present study confirm these findings, since the fish that had exhibited compensatory growth had significantly poorer sustained swimming performance than *ad libitum* fish. However, it was surprising to find that dietary supplementation of resveratrol and carotenoids had no effect on swimming performance, despite their presumed beneficial effect on the antioxidant system (Yeum et al. 2009; Frombaum et al. 2012). Although, it is less surprising given the lack of differences in GPx activity and protein carbonyl levels found in this experiment between compensatory growth and *ad libitum* growth fish. It may be that the resources invested in compensatory growth may have been diverted away from important components related to swimming performance such as muscle strength and function which could not be restored by the supplementation of dietary antioxidants. Indeed, muscle growth-related genes and the development of muscle fibres are known to be negatively affected by accelerated growth after periods of fasting (Galloway et al. 1999; Nebo et al. 2013). Regardless, an alternative antioxidant supplement (tocotrienol-rich fraction TRF), has been successfully shown to improve forced swimming in Male Wistar rats (Lee et al. 2009).

2.5.4 Oxidative stress status

After 22 weeks of supplementation, resveratrol-fed fish had a significantly reduced superoxide dismutase (SOD) activity in comparison with fish not fed resveratrol. SOD is the first line of defence against free radicals produced by lipid peroxidation that are derived from oxygen. The decrease in SOD indicates a down-regulation of the defence mechanism which copes with the production of superoxide anions. One interpretation of this could be that resveratrol is working as an alternative (non-enzymatic) antioxidant and is therefore able to defend against high levels of oxidative stress produced through compensatory

growth. For instance, in order to achieve no changes in protein carbonyls (which is an indicator of oxidative damage to proteins), resveratrol-fed fish may not have required to upregulate SOD antioxidant enzyme defences to the same extent as the fish that had not received resveratrol. This is an interesting result, as an increase in the investment of the enzymatic antioxidant system (in terms of upregulation of SOD in this case) in response to compensatory growth can only come at a cost to investment elsewhere. Therefore, potentially other life history components could be compromised which could subsequently affect fitness in different ways. However, a previous study investigating swimming endurance capacity in male Wistar rats found that the TRF-treated rats were able to swim significantly longer, had lower levels of muscle protein carbonyls than controls and increased levels of GPx and SOD activity (Lee et al. 2009). Taken together, these results suggest that perhaps resveratrol may not have been an effective antioxidant in this experiment.

It would have also been interesting to have obtained the oxidative stress results for the fish lost in the freezer accident that were culled at the end of Period 1, when the C growth regime fish had began their growth compensation period. These results would have given insight into the oxidative stress status of the fish at this crucial and highly energetic time, and could have provided evidence of whether or not compensatory growth does indeed increase levels of oxidative stress. The timing of the final oxidative stress measurements, 15 weeks after the food restriction had been uplifted, may have reduced the likelihood of detecting differences in levels of protein carbonyl content between treatments. By this point, the C growth regime fish may have fully repaired any oxidative damage that had developed during the period of growth compensation.

2.5.5 Conclusions

Overall, the beneficial effects of resveratrol reported in the present study exemplify and make progressive advances in determining the importance of dietary antioxidants in mediating trade-offs. However, these benefits are far less extreme than what has been demonstrated in previous studies that have investigated the effects of resveratrol in a less complete life history setting (for example such as in Valenzano et al. (2006) and Yu et al. (2012), where the fish were kept in an unnatural situation with unlimited access to food). The present results, based on a vertebrate species not previously studied in this context, provide a bone of contention to the breadth of studies (mostly in lower-order animals) that

proclaim resveratrol is a powerful antioxidant which delays ageing, such as in the budding yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, and in the fruit fly *Drosophila melanogaster* (Howitz et al. 2003; Wood et al. 2004; Bauer et al. 2004; Viswanathan et al. 2005).

CHAPTER 3 – DIETARY RESVERATROL AND CAROTENOIDS INFLUENCE OXIDATIVE STRESS STATUS AND CLUTCH MASS BUT NOT CLUTCH ANTIOXIDANT CAPACITY

3.1 ABSTRACT

This chapter demonstrates that compensatory growth occurs when a food restriction in early life is subsequently uplifted allowing the three-spined sticklebacks to achieve a normal body size in time for the breeding season. However, it has been established that compensatory growth can have negative effects on traits later in life. It was predicted that as accelerated growth is metabolically demanding, females that have invested their resources in undergoing this growth strategy may exhibit a reduced investment in their reproductive output which is also energetically expensive. The high metabolic costs of compensatory growth and producing offspring are suggested to be associated with an increase in the production of reactive oxygen species, which can be damaging when they are able to accumulate to certain levels. Dietary antioxidants are suggested to play a role in combating oxidative stress. Therefore, it was predicted that two potential antioxidants, resveratrol and carotenoids would play a beneficial role in ameliorating oxidative stress when supplemented in the diet of male and female mature sticklebacks. Indeed, the results showed that the oxidative damage the sticklebacks incurred was influenced by their relative intake of carotenoids, with a diet higher in carotenoids significantly reducing oxidative damage to proteins. Additionally, it was anticipated that the oxidative stress benefits of these dietary supplements would consequently lead to improvements in female breeding investment and increased antioxidant capacity in the mothers' eggs. In line with predictions, females supplemented with a diet high in carotenoids had larger clutches suggesting that carotenoids played a positive role in female egg production. However, this was only apparent when the fish had not also been supplemented with resveratrol, suggesting that resveratrol may have imposed a detrimental effect on egg production. Overall this chapter shows that carotenoids but not resveratrol play an important role in relieving the oxidative costs associated with energetically demanding activities such as compensatory growth and reproduction.

3.2 INTRODUCTION

3.2.1 Dietary antioxidants

Organisms incur oxidative stress when their rate of production of pro-oxidative by-products of metabolism exceeds the capacity of their antioxidant and repair machinery (Finkel & Holbrook 2000; Metcalfe & Alonso-Alvarez 2010). In this scenario, their antioxidant defence system is unable to completely quench these pro-oxidants and this can result in oxidative damage to biomolecules such as DNA, proteins and lipids (Barja 2004; Hulbert et al. 2007; Pamplona 2011). Accumulated oxidative damage can have negative effects on important biological processes and can result in degenerative diseases and eventually death of the organism (Finkel & Holbrook 2000; Hulbert et al. 2007). The expression of many life history traits such as growth rate, fecundity and the elaboration of sexual ornaments have been found to be negatively affected by high levels of oxidative stress (von Schantz et al. 1999; Alonso-Alvarez et al. 2007; Bize et al. 2008; De Block & Stoks 2008). Resistance to oxidative stress has also been suggested to be a predictor of survival (Selman et al. 2012).

Dietary derived antioxidants have been suggested to play a significant role in cellular protection and reducing oxidative stress (Vertuani et al. 2004; Catoni et al. 2008). Due to this, there has been a great wealth of biomedical and biological research investigating how life history traits are influenced by various dietary antioxidants (McGraw & Ardia 2003; Catoni et al. 2008; Pike et al. 2010a; Noguera et al. 2011). Many studies have hypothesised that the supplementation of particular types of dietary antioxidants will support the endogenous antioxidant system, thereby reducing oxidative damage (Poljsak 2011). However, although this is the case in many *in vitro* studies, supplementation trials with dietary antioxidants in animal models have had minimal success (Selman et al. 2006; Perez-Rodriguez 2009; Halliwell 2011).

There has been considerable interest in the role of carotenoids in their ability to protect against oxidative stress (Yeum et al. 2009; Skibsted 2012). However, there has been much debate regarding how important carotenoids are in their role as antioxidants, with the current consensus being that this varies greatly across taxonomic groups (Hartley & Kennedy 2004; Costantini & Møller 2008; Garratt & Brooks 2012). In fact, supplementation with dietary carotenoids was found to increase levels of oxidative stress

by 115% in captive adult kestrels *Falco tinnunculus* suggesting it may have pro-oxidant effects in some scenarios (Costantini et al. 2007a). Carotenoids have been found to exhibit pro-oxidant effects in a number of other studies (reviewed in Yeum et al. (2009)). One suggestion is that carotenoid-based sexual signals are not displaying the antioxidant capacity of the carotenoids themselves; instead they are suggested to reflect the concentrations of other colourless antioxidants (Hartley & Kennedy 2004). These colourless antioxidants may then help mitigate the oxidative decolouration of carotenoids making them available for sexual signalling (Bertrand et al. 2006b; Pérez et al. 2009).

There has been considerable work on non-carotenoid antioxidants in the field of human nutrition (Lettieri-Barbato et al. 2013; Vetrani et al. 2013). However, there has been a lack of investigation into their roles from an ecological approach. Instead, previous ecological research has been biased towards the role of carotenoids which has led to the neglect by ecologists of possibly more effective antioxidants such as the polyphenolic compounds (Catoni et al. 2008). Polyphenols are a more diverse and common class of compounds in comparison with carotenoids, and are therefore potentially more relevant in developing our understanding of the ecological and evolutionary significance of dietary antioxidants (Catoni et al. 2008; Cai et al. 2011). Although powerful antioxidant activities of polyphenols have been established *in vitro* (Spanou et al. 2007; Spanou et al. 2008), mixed effects have been found *in vivo*. Some *in vivo* studies have shown antioxidant effects, others no effects and some have reported mild pro-oxidant effects (Halliwell 2008; Veskoukis et al. 2012). The evidence to support/oppose the antioxidant properties of resveratrol - which is the polyphenolic dietary antioxidant supplemented in the present study - are discussed in Chapter 1.

Major food items of animals such as seeds, fruit, leaves and arthropods contain a suite of dietary antioxidants in numerous combinations (Catoni et al. 2008). Thus, dietary antioxidants are more likely to be ingested in combination in the wild. Therefore, it is imperative to understand the potential physiological roles of dietary antioxidants in combination with one another in reducing oxidative stress (Catoni et al. 2008). In the present study, two carotenoids - lutein and astaxanthin - were supplemented in combination with a polyphenolic compound – resveratrol, in the diet of the experimental three-spined sticklebacks *Gasterosteus aculeatus*. These compounds belong to two different dietary antioxidant classes (Catoni et al. 2008), and therefore differences in their biochemistry may result in positive or negative interactions between them. These

interactions have been little studied in the past (Skibsted 2012). Resveratrol is a hydrophilic compound that is concentrated in tissues such as heart, liver and stomach (Fernández-Mar et al. 2012). In contrast, carotenoids are lipophilic compounds and concentrate in adipose tissue, skin and egg yolk (Surai 2002; Perez-Rodriguez 2009). Here, oxidative stress analyses were carried out on whole body homogenates to account for the differences in this potential dispersion of the different antioxidants amongst tissues.

3.2.2 Increasing oxidative stress through compensatory growth

It has been postulated that fast growth may increase oxidative damage due to the greater mitochondrial activity required for tissue growth, which may consequently result in a greater exposure to reactive oxygen species (ROS) (Alonso-Alvarez et al. 2007; Larcombe et al. 2010a). Fast growth has indeed correlated with oxidative stress (Mangel & Munch 2005; Nussey et al. 2009; Kim et al. 2011). For example, oxidative damage has been found to be positively correlated with growth rate during the first four months of life in the Soay sheep *Ovis aries* (Nussey et al. 2009). However, no relationship between growth rate and oxidative damage was found in the red-winged blackbird *Agelaius phoeniceus* (Hall et al. 2010). Contrasting views such as these may be due to differences in methodological techniques in measuring oxidative stress (Selman et al. 2012). Therefore, it is important in the progression of oxidative stress research that methodological techniques become more standardised between studies.

It has been suggested that oxidative stress may be especially important during the accelerated growth phase of compensatory growth (Mangel & Munch 2005; De Block & Stoks 2008; Lee et al. 2011). In addition, the food restriction period prior to compensatory growth is also associated with higher levels of oxidative stress (Furné et al. 2009). For instance, a 46-day food restriction in gilthead seabream *Sparus aurata* led to a 12.4-fold increase in malondialdehyde levels (MDA), which is extensively used as a biomarker of oxidative damage. Oxidised glutathione levels (GSSG) were also increased which are indicative of a redox imbalance as glutathione is a non-enzymatic antioxidant which plays an important role as a cellular redox buffer (Pascual et al. 2003; Apel & Hirt 2004). A more recent study found that the MDA levels in the tissues of brown trout *Salmo trutta*, increased during both a period of food restriction and after the restriction had been uplifted (Bayir et al. 2011).

A compensatory growth regime was adopted as one of the treatments in the present study to increase the likelihood of oxidative stress endured by the experimental three-spined sticklebacks. As growth is exhibited across all tissues in the body, it has been suggested that polyphenolic compounds (such as resveratrol) may play a particularly important antioxidant role during growth due to their relatively broad potency across tissues in comparison with other dietary antioxidants (Catoni et al. 2008).

3.2.3 Oxidative stress and reproductive investment

It has been suggested in the past that the fitness consequences of compensatory growth are delayed until after reproduction (Metcalfe & Monaghan 2001). However, a recent meta-analysis across diverse taxa has concluded otherwise; achieving a large size through compensatory growth is found to trade off with reproduction, resulting in lower reproductive output (Hector & Nakagawa 2012). For instance, despite three-spined sticklebacks fully compensating in size by the onset of the breeding season, model predictions based on previous experimental observations have suggested that this growth compensation can still result in sub-optimal breeding performance (Lee et al. 2011). This has been proposed to be due to an increased activity level whilst obtaining sufficient food in order to achieve growth compensation, which subsequently causes an increase in the accumulation of damage to important tissues associated with breeding (Lee et al. 2011). However, to date this model prediction has not been tested empirically. In addition, breeding itself is a metabolically demanding activity which has been found to co-vary with resistance to oxidative stress (Nilsson 2002; Bize et al. 2008; Barim 2009; Costantini 2010). In support of this, increased parental effort has been associated with a decrease in resistance to oxidative stress in great tits *Parus major* (Christe et al. 2012). Increased egg production in *Drosophila melanogaster* has also been found to increase susceptibility to oxidative stress, and is thought to be a physiological cost of reproduction (Wang et al. 2001). More recently, in North American red squirrels *Tamiasciurus hudsonicus*, investment in lactation - a high energy expending activity - was found to result in reduced antioxidant defence systems which subsequently led to higher plasma protein oxidative damage (Fletcher et al. 2013). The present study hypothesises that the supplementation of dietary antioxidants would improve oxidative stress status and that these benefits would be reflected in an enhanced female breeding performance. However, there is evidence in the literature to suggest otherwise. For instance, carotenoid availability has not been found to improve the capability of nestling kestrels in contending with oxidative stress (Costantini

et al. 2007b). Despite this, maternally transferred carotenoids have been suggested to play a beneficial antioxidant role in developing embryos (Costantini et al. 2007b). Supplementation trials have found that an increase in the concentration of the carotenoid canthaxanthin in the feed of breeding female chickens significantly increases anti-oxidative status in both chick embryos and post-hatched chicks (Surai et al. 2003; Robert et al. 2008; Zhang et al. 2011; Rosa et al. 2012). The antioxidant properties of canthaxanthin have also been postulated due to its ability to increase female hatching rate, as oxidative stress is known to be an important determinant of hatching success in chickens (Surai 2012). However, it is well regarded in the literature that antioxidant responses are not uniform across taxa and it is therefore important not to generalise these findings in birds to other species which may respond to oxidative challenges in different ways (Costantini et al. 2010). The present study determined whether dietary antioxidant supplementation in female three-spined sticklebacks had a positive impact on egg clutch mass. The study also assessed whether dietary antioxidant supplementation of mothers had a positive impact on their non-enzymatic antioxidant capacity and also that of their clutches. Non-enzymatic antioxidant capacity was measured as opposed to endogenous enzymatic antioxidant capacity, which would not have been an appropriate measurement to use in the case of the unfertilised eggs. These measurements of antioxidant capacity were taken from the mothers and her respective eggs only (e.g. not measured in the mature males).

3.2.4 Measuring oxidative stress in mature sticklebacks

The oxidative balance of living tissues can be divided into three functional components, the mitochondrial production of reactive oxygen species (ROS), the countervailing influence of antioxidant systems (enzymatic and non – enzymatic) and the oxidative damage to bio-molecules (Monaghan et al. 2009). Measuring each of these components is important in order to make appropriate inferences of oxidative stress status (Costantini 2008). For example, many ecological studies in the past have measured only a single component of oxidative stress such as antioxidant capacity (Alonso-Alvarez et al. 2004; Bertrand et al. 2006a; Alonso-Alvarez et al. 2007). However, this provides an inadequate measurement of overall oxidative stress status by over-emphasising one aspect of oxidative stress and neglecting other important components (Costantini & Verhulst 2010). For example, a common hypothesis is that high antioxidant enzyme activities reflect a high level of oxidative stress (Montgomery et al. 2011). However, this is not necessarily the case, as utilising information on antioxidant enzymes alone is insufficient in demonstrating

oxidative stress (Costantini & Verhulst 2010). Superoxide dismutase (SOD) and glutathione peroxidase (GPx) are two important endogenous antioxidant enzymes that play a role in combating ROS. The present study therefore measured levels of SOD and GPx in both the male and female mature sticklebacks as two measures of endogenous antioxidant enzyme activity. In addition, protein damage was assessed by measuring protein carbonyl concentrations which is a widely utilised measure of protein oxidation (Dalle-Donne et al. 2003). Protein damage was measured in both the male and female mature sticklebacks.

3.2.5 Aims

The aim of the present study was to investigate the extent to which resveratrol, carotenoids and compensatory growth affected three-spined sticklebacks in two important components of oxidative stress: antioxidant enzyme activity levels and oxidative damage. The assays used in this study provided a comprehensive overview of the oxidative stress status of the sticklebacks fed with different combinations of resveratrol and carotenoid supplements. The study also investigated the effects of resveratrol, carotenoids and growth trajectory on female stickleback clutch mass. Therefore, it was possible to determine whether greater investment in egg production came at a cost for self maintenance which was hypothesised to be reflected in increased oxidative stress in the adult females.

3.3 METHODS

3.3.1 Source of fish and rearing conditions

Underyearling three-spined sticklebacks were collected on 17th January 2011 following the same procedures described in Chapter 2. Following capture the fish were transported to the University of Glasgow and transferred to acclimatisation aquaria (45-L and density 3 fish L^{-1}) for four weeks and fed *ad libitum* (i.e. 10% of body mass/day) with frozen chironomid larvae until the experiment commenced. The fish were kept under the same husbandry procedures detailed in Chapter 2.

On 17th February 2011, all 256 fish were anaesthetised and measured for standard length ($\pm$0.01 mm) and wet mass ($\pm$0.001g). All fish were then randomly-selected and assigned into pairs of differing size in order to aid identification of individuals. Each pair of fish was transferred into a separate (7-L) tank (33 × 18 × 19 cm). Males were further separated into

their own individual (7-L) tank when they had developed their first signs of breeding colouration.

3.3.2. Growth and dietary supplement manipulations

The 256 fish were allocated to 8 feeding treatments which are described in Table 2.1 in Chapter 2. The feeding treatments began on 17th February 2011. Half of the fish (i.e. 64 tanks of 2 fish) were randomly assigned to the compensatory (C) growth regime, and the remainder to the *ad libitum* (A) growth regime. The restricted food ration was applied for a three week period (Period 1); by this time point the C growth regime fish were significantly smaller in size in comparison with the A growth regime fish. This restricted ration was then uplifted on 10th March 2011 to induce compensatory growth in the C growth regime fish (Period 2).

The 64 tanks within both the C and A growth regimes were further randomly assigned into 16 replicate tanks for each of the four dietary supplement manipulations. These dietary supplement manipulations are described in Section 2.3.3 in Chapter 2. Thus, overall within this experiment there were 8 feeding treatments (2 growth regimes × 2 resveratrol regimes × 2 carotenoid regimes, each with 16 replicate tanks containing 2 fish), allowing evaluation of the effect of dietary restriction-induced compensatory growth on oxidative stress status in mature sticklebacks as well as on the antioxidant capacity of the females' eggs (Figure 3,1). In addition, it could also be determined whether dietary supplements of resveratrol and carotenoids influenced any of these responses.

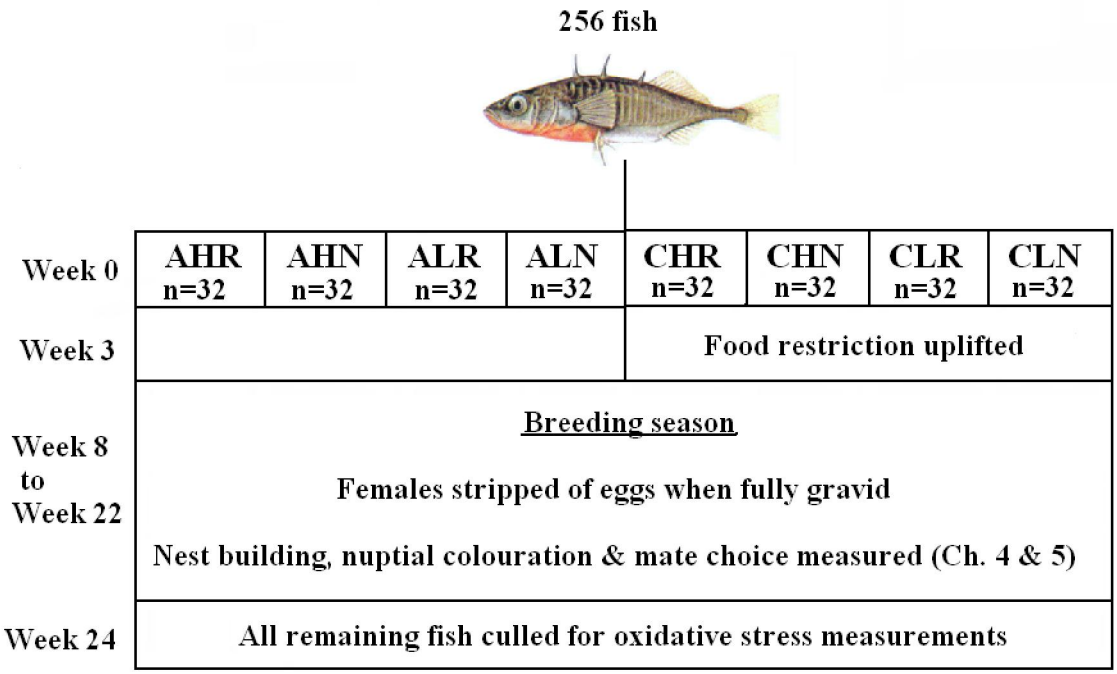


Figure 3.1 Schematic of experimental design. Fish were equally divided into the eight feeding treatments described in (Table 2.1, Chapter 2). At week 3 the food restriction was uplifted in the four compensatory growth regime fish (CHR, CHN, CLR and CLN). At week 8, the breeding season had commenced and females were observed daily and stripped when fully gravid. At week 24, all remaining fish were culled for oxidative stress measurements.

There was an unusually high mortality rate during the initial phase of the experiment. Immediate attempts were made to determine and rectify this. For example, good water quality was assured using both ammonia and nitrate test kits. All fish were also treated with Methylene blue, a common "first response" treatment for sick fish. This was added to the tank water along with an increase of salt. Samples of fish were also examined by a veterinary clinical officer. Microscopic examinations revealed various body surface lesions and evidence of white spot *Ichthyophthirius multifiliis, Monogenea* flukes and *Saprolegnia* fungus. The veterinary officer advised that the remaining live fish should be treated with Malachite Green at a concentration of 0.1ml/10l of tank water to attempt to resolve this problem. Unfortunately Malachite Green only alleviated the mortality rate as 38.3% of the experimental fish died during the experiment. It should be acknowledged that unfortunately the mortality at the start of the experiment may have caused an *a priori* selection in favour of the strongest fish which may have potentially masked some underlying patterns in the present study.

3.3.3 Egg and fish sample preparations

All females were observed daily throughout the breeding season to determine when they appeared to be fully gravid (i.e. had a grossly distended abdomen). The gravid females were then anaesthetised using benzocaine and stripped of eggs following the protocol of Ali and Wootton (Ali & Wootton 1999). Prior to being stripped of their eggs, a random selection of 32 fully gravid females were used in the mate choice trials described in Chapter 5. All females were then weighed after the stripping procedure and then returned to their experimental tanks after a 10 minute recovery period. The egg clutches were saved in 100% ethanol in Eppendorf vials and immediately stored at -80°C in the dark until being homogenised for non-enzymatic antioxidant capacity analyses.

On 31st August 2011, once the breeding season had ceased, all fish were culled, weighed, measured, snap-frozen in liquid nitrogen and stored in darkness at -80°C until they were homogenised in preparation for the oxidative stress analyses.

In order to prepare the egg samples for the non-enzymatic antioxidant capacity analysis, the ethanol that the eggs were stored in was evaporated using a centrifugal evaporator. However, a degradation test was first carried out using an OXY-adsorbent reference standard (Diacron International) in order to validate that the egg samples were unaffected by the evaporation process. A known concentration of OXY-adsorbent reference standard was diluted with 100% ethanol, in quadruplicate. These four samples were then placed in a centrifugal evaporator for 30 minutes in order to determine whether the known volume of standard remained post-evaporation of the ethanol. The evaporated samples were then analysed using an OXY-adsorbent test kit following the methodologies described in Section 3.3.5. This degradation test obtained an extraction efficiency of 99.6%. Therefore, it could be disregarded that the evaporation process caused any degradation to the egg samples. Immediately after the evaporation process, the dry mass of each egg clutch was recorded to the nearest 0.0001g. The fish samples were removed from the freezer and left to thaw for one hour.

The egg and fish samples were then diluted in ice-cold phosphate buffer saline (0.01 mol pH 7.0) with 20% glycerol and 0.2mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, UK) at a dilution factor of 1:10 and 1:4, respectively. Separately, the samples were homogenised using a Potter Elvehjem homogeniser and then centrifuged, in an Eppendorf

centrifuge 5417C for 15 minutes at a speed of 10,000 rpm, at 4°C. The supernatants were then pipetted into Eppendorf vials. The centrifuging process was repeated twice to remove any remaining solid masses of egg or fish. The supernatant samples were then aliquoted into 1.5ml Eppendorf vials and immediately stored at -80°C in the dark until all samples had been processed and were ready for oxidative stress analyses.

3.3.4 Measuring total protein content in mature sticklebacks

A Quick Start ^{TM} Bradford assay kit (Bio-Rad Laboratories, Inc. California) was used to measure the total protein content of each of the stickleback and egg samples. The total protein content values for each sample could then be used to standardise for the measurements carried out in the Ransod, Ransel, OXY-adsorbent, and protein carbonyl assays. Prior to the analysis, the Bradford dye reagent from the test kit and one vial of each of the stickleback samples and egg samples were left out of the fridge and freezer for an hour to equilibrate to room temperature. To calibrate this assay, a serial dilution was made using a bovine serum albumin (BSA) protein standard (Bio-Rad Laboratories, Inc. California) of known concentration (Table 3.1).

The stickleback and egg samples were diluted in distilled water (dH_2O) in a ratio of 1:50 (e.g. 2µl of sample in 98µl of dH_2O). Then, 5µl of each of the protein standard dilution series (A-E), the blank and the diluted samples were pipetted into each well of a Corning Costar 96-well flat bottomed plate, in duplicate. Using a multichannel pipette, 200µl of Bradford dye reagent was added to each well. At this point, a timer was immediately started. After 10 minutes, the reaction was complete and the absorbance of each well was read at 595nm by a Thermo Scientific Multiskan Spectrum (ThermoFisher, Vantaa, Finland). The intra-assay coefficient of variation was 4.29%. The values of protein content were divided by 0.25 and 0.1 to correct for the original stickleback and egg clutch dilutions of 1:4 and 1:10, respectively.

Table 3.1 The dilution series of the BSA protein standard in dH_2O which was used to calibrate the Quick Start ™ Bradford assay.

Standard	Volume of standard of	Volume of water
A	100%	-
B	100µl of A	100µl
C	100µl of B	100µl
D	100µl of C	100µl
E	100µl of D	100µl
F	-	100%

3.3.5 Measuring antioxidant capacity in egg clutches and the stripped females

An OXY-adsorbent test (Diacron International) was used as a measure of the non-enzymatic antioxidant capacity of both the female sticklebacks and egg clutch homogenates using the following protocol. This test measures the hydrophilic and lipophilic antioxidant action of the plasmatic barrier by measuring its ability to oppose the oxidative action of hypochlorous acid (HClO). The test assesses this colorimetrically by measuring the residual unreacted acid.

Prior to the analysis, the reagents from the test kit and one vial of each of the stickleback samples and egg samples were left out of the fridge and freezer for an hour to equilibrate to room temperature. The reagents from the test kit were comprised of the oxidant solution which is HOCl- based (R_1 reagent), chromagen (N, N-diethylparaphenilendiamine) (R_2 reagent) and the reference standard (R_3 reagent). The fish and egg samples were diluted with dH_2O in a ratio of 1:200 and vortexed for 30 seconds. Next, the R_3 reagent was diluted in dH_2O in a ratio of 1:100 and also vortexed for 30 seconds. Then, 2µl of each diluted sample, the diluted R_3 reagent and the blank (dH_2O) were pipetted into each well of a Corning Costar 96-well flat bottomed plate, each in duplicate. A multichannel was then used to pipette 200µl of the R_1 reagent into each well. The plate was then incubated for 10 minutes at 37°C with mild shaking (60pM) in a Thermo Scientific Multiskan Spectrum which was programmed using Thermo Scientific SkanIt 2.4.4 software. At the end of incubation the plate was removed, and 2µl of R_2 reagent was pipetted into each well using

a multichannel. The well contents were then mixed using the multichannel. The absorbance of each well of the plate was then read at 490nm. The OXY value was then calculated using the following formula:

[(Absorbance of blank – Absorbance of sample) / (Absorbance of blank - Absorbance of R_3 reagent)] × (R_3 reagent OXY value)

Each sample's OXY value was then multiplied by 200 to account for the initial sample dilution factor of 1:200. The R_3 reagent OXY value was also corrected for the initial dilution factor of 1:100. The intra-assay coefficient of variation was 6.04%. Next, the sample's OXY values were divided by 0.25 or 0.1 to correct for the original stickleback and egg clutch dilutions of 1:4 and 1:10, respectively. The values were then corrected to account for the total protein content per sample which had been measured in a separate analysis (See Section 3.3.4). The OXY values could then be expressed as µmol mg protein/ g^{-1} fish or egg of neutralised HOCl.

3.3.6 Measuring Superoxide dismutase activity in mature sticklebacks

A Superoxide dismutase (SOD) assay kit (Ransod, Randox Laboratories, Crumlin, UK) was used for the quantitative *in vitro* determination of SOD activity in the mature male and female stickleback samples. SOD is an endogenously derived antioxidant enzyme which is responsible for scavenging the toxic superoxide radical (McCord & Fridovich 1969). The reagents from the test kit and one vial of each of the stickleback samples were left out of the fridge for an hour to equilibrate to room temperature. The test kit was comprised of a mixed substrate (xanthine and 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) (R_{1a} reagent), buffer (R_{1b} reagent), xanthine oxidase (R_2 reagent) and a reference standard (R_3 reagent). These reagents were prepared according to the instructions found in the Ransod Randox Laboratories manual provided with the test kit. Ransod diluent and Ransod quality control (Randox Laboratories Ltd.) were also left to equilibrate to room temperature.

The assay employs the mixed substrate (R_{1a} reagent) and xanthine oxidase (R_2 reagent) to generate superoxide radicals. These superoxide radicals then react with the I.N.T to produce a red formazan dye. The superoxidase activity is then calculated by the degree to which each fish sample is able to inhibit this reaction. This is measured colorimetrically

using a Thermo Scientific Multiskan Spectrum. The assay was adapted by Dr. David Costantini in order to use Corning Costar 96-well flat bottomed plates rather than cuvettes to save both reagents and time. These plates were also more suited for working with the volumes of stickleback samples. The serial dilutions required to produce a standard curve were also modified along with the quantities of diluted sample required in the procedure. This ensured that the stickleback samples fitted the linearity of the assay (Table 3.2). The modified calibration dilution series appropriate for the stickleback samples can be found in Table 3.2. The stickleback samples were further diluted 1:40 with Ransod diluent before analysis. The Ransod quality control was reconstituted with 1ml of dH_2O, swirled gently, left for 15 minutes, and then diluted 1:800 with Ransod diluent.

Then, 6µl of each of the standard dilution series (A-G), the diluted stickleback samples, the Ransod quality control and the blank (H; 100% Ransod sample diluent) were pipetted into each well of the plate, each in duplicate. No more than three rows of a plate were tested at any one time. Next, a multichannel was used to pipette 200µl of the R_{1a} reagent into each well. The wells were then mixed using the multichannel. Then, 12.5µl of the R_2 reagent was added to each well using a multichannel. A timer was immediately started at this point. The plate was then inserted into the Thermo Scientific Multiskan Spectrum and the initial absorbance at 505nm was read at a time point 30 seconds after the Xanthine Oxidase had been added to the sample (A_1). The plate content was then mixed well using a multichannel and a final absorbance was measured 3 minutes after the Xanthine Oxidase had been added to the sample (A_2). The change of absorbance per minute in each sample or standard was calculated using the formula:

$(A_2 - A_1)/ 3 =$ ΔA/min of standard or sample

The following equation was then used to calculate the percentage of inhibition of the reaction in each of the stickleback samples and standards:
100 - (Δstd or sample/min × 100) / (Δblank/min) = % inhibition

Next, the percentage of inhibition for each standard dilution was plotted to create a standard curve. The percentage of inhibition for each fish sample was then plotted on the standard curve to obtain the measurement of SOD activity.

The intra-assay and inter-assay coefficients of variation were 4.92% and 1.42%. The values were divided by 0.25 to correct for the original 1:4 stickleback dilution during homogenisation. The values were then corrected to account for the total protein content per sample. The concentration of SOD could then be expressed as Units/mg protein/gram of fish.

Table 3.2 The dilution series of Ransod standard (R_3 reagent) in Ransod sample diluent (0.01 mol/l phosphate buffer, pH 7.0) which was used to calibrate the Ransod assay.

Standard	Volume of standard solution of	Volume of sample diluent
A	Undiluted R_3 reagent	-
B	200µl of A	200µl
C	200µl of B	200µl
D	200µl of C	200µl
E	100µl of D	50µl
F	100µl of D	100µl
G	100µl of F	50µl
H	-	100% sample diluent

3.3.7 Measuring Glutathione peroxidase activity in mature sticklebacks

A Glutathione peroxidase (GPx) assay kit (Ransel, Randox Laboratories, Crumlin, UK) was used for the quantitative *in vitro* determination of GPx activity in the adult male and female stickleback samples. GPx is an endogenously derived antioxidant enzyme which catalyses the reduction of hydrogen peroxide and hydroperoxides to water and to their respective alcohols, respectively, using glutathione as a cofactor (Paglia & Valentine 1967).

The reagents from the test kit and one vial of each of the stickleback samples were left out of the fridge and freezer for an hour to equilibrate to room temperature. The test kit was comprised of a reagent which consisted of glutathione, glutathione reductase and NADPH (R_{1a} reagent), buffer (R_{1b} reagent), cumene hydroperoxide (R_2 reagent) and a diluting agent (R_3 reagent). These reagents were prepared according to the instructions found in the

Ransel Randox Laboratories manual provided with the test kit. A vial of Ransel quality control (Randox Laboratories Ltd.) was reconstituted with 1ml of dH_2O. In preparation for analysis, 25µl of this Ransel quality control was added to 1ml of (R_3 reagent) and vortexed for 30 seconds.

The assay employs the R_{1a} reagent and R_2 reagent together, which catalyses the oxidation of glutathione. The presence of the glutathione reductase and NADPH in the R_{1a} reagent allows the oxidised glutathione to be immediately reduced. This glutathione peroxidase activity is then measured as the decrease in absorbance caused by this conversion of glutathione to its reduced form. This is measured using a Thermo Scientific Multiskan Spectrum. This assay was also adapted by Dr. David Costantini in order to use Corning Costar 96-well flat bottomed plates rather than cuvettes to save both reagents and time. In this assay, the stickleback samples were maintained in their original 1:4 dilution. To begin, 200µl of the R_{1a} reagent was pipetted into each well of the plate using a multichannel. No more than three rows of a plate were tested at any one time. Then, 4µl of each of the stickleback samples and the Ransel quality control were pipetted into each well, each in duplicate. A multichannel was then used to pipette 8µl of the R_2 reagent into each well. The wells were then mixed using the multichannel. The plate was then inserted into the Thermo Scientific Multiskan Spectrum and the initial absorbance at 340nm was read immediately (A_1). A timer was immediately started at this point. The absorbance reading was repeated twice more, 1 minute (A_2) and 2 minutes (A_3) after the cumene hydroperoxide had been added to the sample. To calculate the glutathione peroxidase (GPx) activity in each of the stickleback samples and the quality control the following equation was used:

GPx active units/l of sample or quality control = $15873 \times \Delta A$ at 340nm/minute

The intra-assay and inter-assay coefficients of variation were 5.89% and 11.25%, respectively. The values were divided by 0.25 to correct for the original 1:4 stickleback dilution during homogenisation. The values were then corrected to account for the total protein content per sample analyses separately in Section 3.3.4. The concentration of GPx could then be expressed as Units/mg protein/gram of fish.

3.3.8 Measuring protein carbonyl content in mature sticklebacks

The following protocol measures protein carbonyl content, which is the most widely measured marker of oxidative damage to proteins (Dalle-Donne et al. 2003). The adult male and female stickleback samples were diluted with dH_2O, in order to obtain a concentration of 1mg of proteins per ml, and then vortexed for 30 seconds. It was important to choose a quantity of sample that had a final volume of no less than 100µl. Next, 10% solution of streptomycin sulfonate was added to each sample using a dilution factor of 1:9 (e.g. 12µl of 10% streptomycin sulfonate solution was added to 108µl of sample). The samples were then left to equilibrate to room temperature for 15 minutes. This step in the protocol was necessary to precipitate and remove any nucleic acids which could have carbonyl groups that could confound with the results of this assay. The samples were then centrifuged at 13,000 rpm for 10 minutes. The supernatant was then removed. Then, 25µl aliquots were made of each the supernatant samples, in duplicate. Next, 400µl of 0.005M DNPH ethanolic solution with 0.1M of HCl (Sigma-Aldrich, UK) was added to each aliquot. Given that variation among all blanks of all samples was really low (1-2%), analysis of 2-4 blanks in each assay was deemed satisfactory. The blank reaction was made by adding 400µl of HCl 0.1M (diluted HCL 2M in ethanol) to 25µl of any 4 extra samples.

The samples and blanks were then vortexed and incubated for one hour at room temperature in the dark to allow for the reaction. The samples were vortexed 30 minutes into this reaction. At the end of incubation, 20% cold trichloroacetic acid was added to the samples and blanks in a ratio of 1:1 (i.e. 425µl). The samples were then vortexed for a few seconds and then centrifuged for 10 minutes at 13,000 rpm. Almost all of the supernatant was then removed and discarded. Care was taken not to damage or remove the intact residue in pellet form at the bottom of the vials. In order to wash the pellet, a 200µl solution (1:1 of cold ethanol-ethyl acetate) was added to the vial and vortexed for a few seconds. The vials were then centrifuged for two minutes at 13,000 rpm. Again, most of the supernatant was removed and discarded. This washing procedure was repeated three times. However, on the last wash the vial was centrifuged for two minutes at 13,000rpm but not vortexed. Afterwards, the vials were left open in a fume cupboard for five minutes in order to dry the pellet as much as possible. Then, 350µl of guanidine hydrochloride (6M) was added to each vial and vortexed for a few seconds. The samples were then covered with tin foil and incubated for 15 minutes at 37°C in a Stuart Scientific Orbital incubator S150 at a shaking speed of 150 rpm.

At the end of incubation, the samples were centrifuged for 2 minutes at 13,000 rpm. Then, 300µl of each sample and blank were pipetted into each well of a Corning Costar 96-well flat bottomed plate. The absorbance of each of the samples was then read at 370nm by a Thermo Scientific Multiskan Spectrum. The concentration of protein carbonyls in each sample was calculated by subtracting the average absorbance of the blanks from the average absorbance of each of the samples. This value was divided by the extinction coefficient for DNPH, which is 0.022/µM/cm (i.e. a solution with a concentration of 1µM has an absorbance of 0.022). The following formula was then used to calculate the total content of protein carbonyls in each sample:

[(Absorbance of sample - Absorbance of blank)/0.022] ×14 (14 is the dilution factor = 350µl/25µl).

The intra-assay coefficient of variation was 4.44%. The values were divided by 0.25 to account for the original 1:4 stickleback dilution during homogenisation. The concentration could then be expressed as nmol/mg of protein.

3.3.9 Statistical analysis of growth

The fish were measured at the beginning of the experiment before growth manipulation commenced, once during the growth manipulation period (Period 1) and three times after the restricted ration had been uplifted (Period 2). It was possible to identify and track the growth trajectory of each individual fish. Therefore, the standard body length and mass of each individual fish from each tank at each sampling point were used as the variables to analyse growth. In order to prevent bias in the experimental results due to mortality, statistical analyses on growth were based on the 158 fish (82 females and 76 males) that survived the duration of the experiment. Differences in standard body length and mass between the *ad libitum* and compensatory growth regimes were tested using a multivariate analysis of variance (MANOVA), as were differences in standard body length and mass between all eight feeding treatments.

3.3.10 Statistical analyses of SOD, GPx and protein carbonyls

The effects of growth regime and dietary supplementation on three elements associated with oxidative stress (SOD activity, GPx activity, protein carbonyl content) were analysed

separately using general linear mixed models (GLMMs), with growth regime (A or C), carotenoid supplementation (H or L), resveratrol supplementation (R or N) and sex as fixed effects, final body length and mass as covariates and tank number as a random factor, plus all two-way interactions among variables. SOD, GPx, and protein carbonyls data were all positively skewed and log transformed prior to statistical analysis. To identify relationships between oxidative stress measurements, a GLMM was constructed with GPx as the dependent variable and SOD and protein carbonyl content as covariates alongside tank as a random factor.

3.3.11 Statistical analyses of non-enzymatic antioxidant capacity in females and their clutches

The effects of maternal non-enzymatic antioxidant capacity (maternal OXY), maternal growth regime and maternal dietary supplementation on clutch non-enzymatic antioxidant capacity (clutch OXY) were analysed using a GLMM, with growth regime (A or C), carotenoid supplementation (H or L) and resveratrol supplementation (R or N) as fixed effects, body length of female at time of spawning, post-stripped body mass, clutch mass and maternal OXY included as covariates and tank number as a random factor, plus all two-way interactions among variables. Maternal OXY, clutch OXY, clutch mass and fish length data at time of stripping were all positively skewed and therefore log transformed prior to statistical analysis.

3.3.12 Statistical analyses of clutch mass

The effect of growth regime, dietary supplementation and maternal oxidative stress status on clutch mass in females was analysed using a GLMM, with growth regime (A or C), carotenoid supplementation (H or L), resveratrol supplementation (R or N) as fixed effects, SOD activity, GPx activity, protein carbonyl content, body length at time of spawning and post-stripped body mass as covariates and tank number as a random factor, plus all two-way interactions among variables. Clutch mass data and fish length at time of stripping were positively skewed and therefore log transformed prior to statistical analysis.

All means are described with standard errors and all analyses were carried out using R (R Core Development Team, version 2.15.0). The function lme within the nlme package was used to fit all the GLMMs. Significant results were defined as $p < 0.05$. Non-significant

variables were sequentially dropped from each analysis so that the final models only included significant terms apart from main effects that occurred in significant two-way interactions.

3.4 RESULTS

3.4.1 Compensatory growth response after food restriction

At the start of the experiment, there were no differences in either the standard length or mass between the two growth regime groups (A and C) (MANOVA: Wilk's $\lambda = 0.992$, $F_{2,155} = 0.633$, $p = 0.53$; Figure 3.2), or among the eight feeding treatments overall (see Table 2.1 in Chapter 2 methods for description of treatment groups) (MANOVA: Wilk's $\lambda = 0.888$, $F_{14,298} = 1.301$, $p = 0.21$). However, after the 3-week manipulation of growth regimes (end of Period 1) there were significant differences in both standard length and mass between the two growth regime groups (A and C) (MANOVA: Wilk's $\lambda = 0.947$, $F_{2,155} = 4.298$, $p = 0.015$). At this time point, the C fish were 4.55% shorter (ANOVA: $F_{1,156} = 6.109$, $p = 0.015$) and 15.3% lighter (ANOVA: $F_{1,156} = 8.355$, $p = 0.004$) than A fish.

There were no effects of resveratrol supplementation on size at the end of period 1 within the A growth regime (MANOVA: Wilk's $\lambda = 0.940$, $F_{2,91} = 2.915$, $p = 0.059$) or within the C growth regime (MANOVA: Wilk's $\lambda = 0.960$, $F_{2,61} = 1.277$, $p = 0.29$). There was also no effects of carotenoid supplementation on size at the end of Period 1 within the A growth regime (MANOVA: Wilk's $\lambda = 0.935$, $F_{2,91} = 3.182$, $p = 0.056$) or within the C growth regime (MANOVA: Wilk's $\lambda = 0.987$, $F_{2,61} = 0.390$, $p = 0.68$).

While there was a borderline significant difference in mean standard length and mass at the end of Period 1 among the eight feeding treatments overall (MANOVA: Wilk's $\lambda = 0.855$, $F_{14,298} = 1.737$, $p = 0.048$), this was really just a consequence of the difference between the two growth regime groups, since within each of these there were no effects of resveratrol supplementation or carotenoid supplementation.

The differences in size began to disappear once the C growth regime fish were transferred onto the A growth regime diet, and after 6 weeks full compensation had occurred as there were no longer significant differences in size between the two growth regime groups (A

and C) (MANOVA: Wilk's $\lambda = 0.976$, $F_{2,155} = 1.944$, $p = 0.15$) (Figure 3.2) or among the eight feeding treatments overall (MANOVA: Wilk's $\lambda = 0.929$, $F_{14,298} = 0.795$, $p = 0.68$).

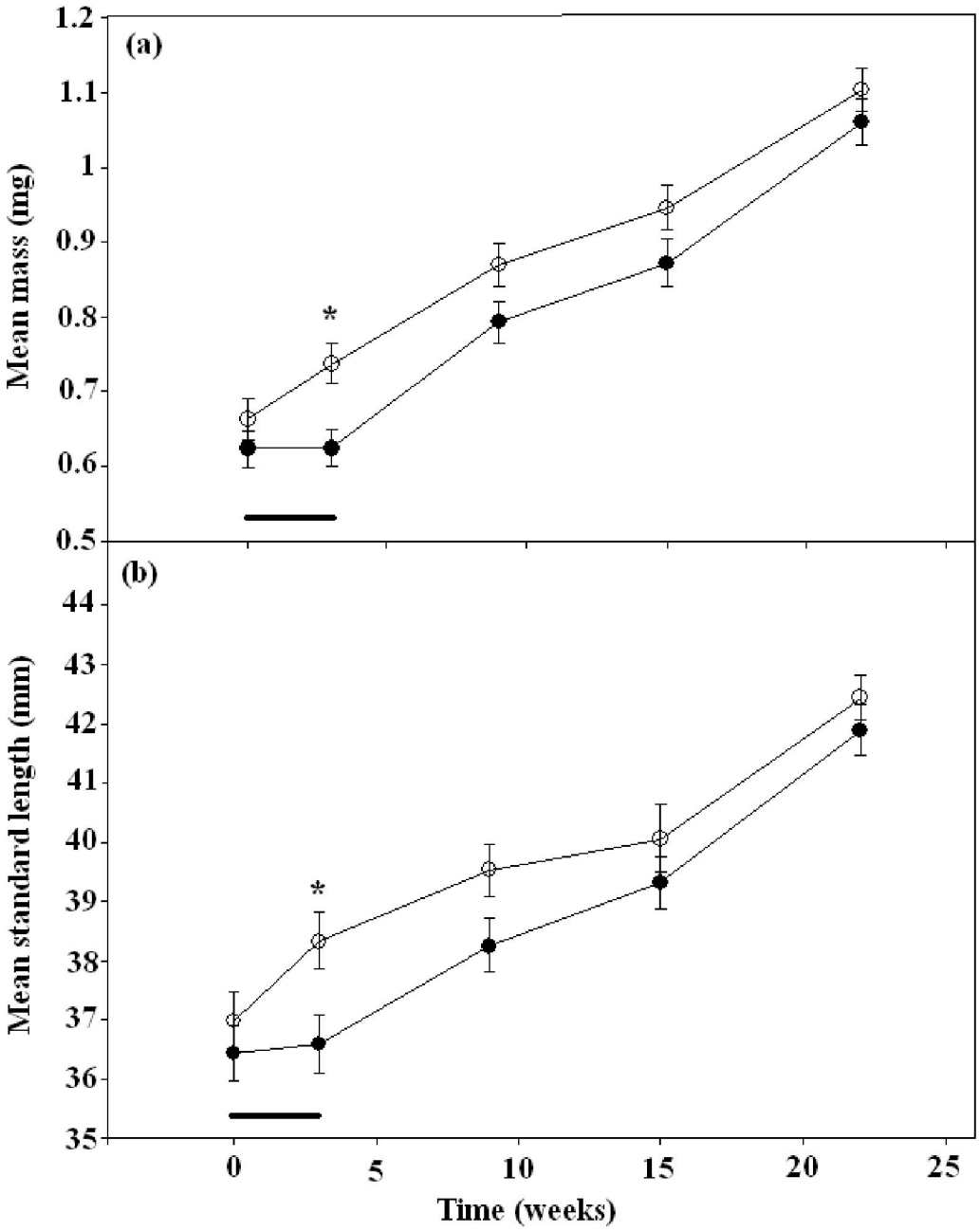


Figure 3.2 Growth trajectories for mean ± s.e. (a) mass and (b) standard length of three-spined sticklebacks in relation to growth regime (*ad libitum*, open circles; compensatory growth, closed circles). Dietary carotenoid and resveratrol supplementation groups are combined to illustrate the effect of growth regime only. The thick horizontal line indicates the period of growth manipulation (3 weeks), after which point all fish were fed *ad libitum* but were still maintained on their original dietary supplementations. Asterisks indicate significant differences in mass or length between the A and C growth regime groups ($p<0.05$); n = 158 fish.

3.4.2 Protein carbonyl content

The fish that were fed a low carotenoid diet had significantly higher protein carbonyl content than fish fed a diet high in carotenoids (GLMM, $t_{109} = 2.695$, $p = 0.008$) (Figure 3.3, Table 3.3). There was no difference in protein carbonyl content in relation to growth regime (GLMM, $t_{106} = 0.077$, $p = 0.94$) or whether resveratrol was present in the diet (GLMM, $t_{107} = 0.678$, $p = 0.50$). Sex did not have any significant effect on protein carbonyl content (GLMM, $t_{108} = -1.580$, $p = 0.18$) (Figure 3.3, Table 3.3).

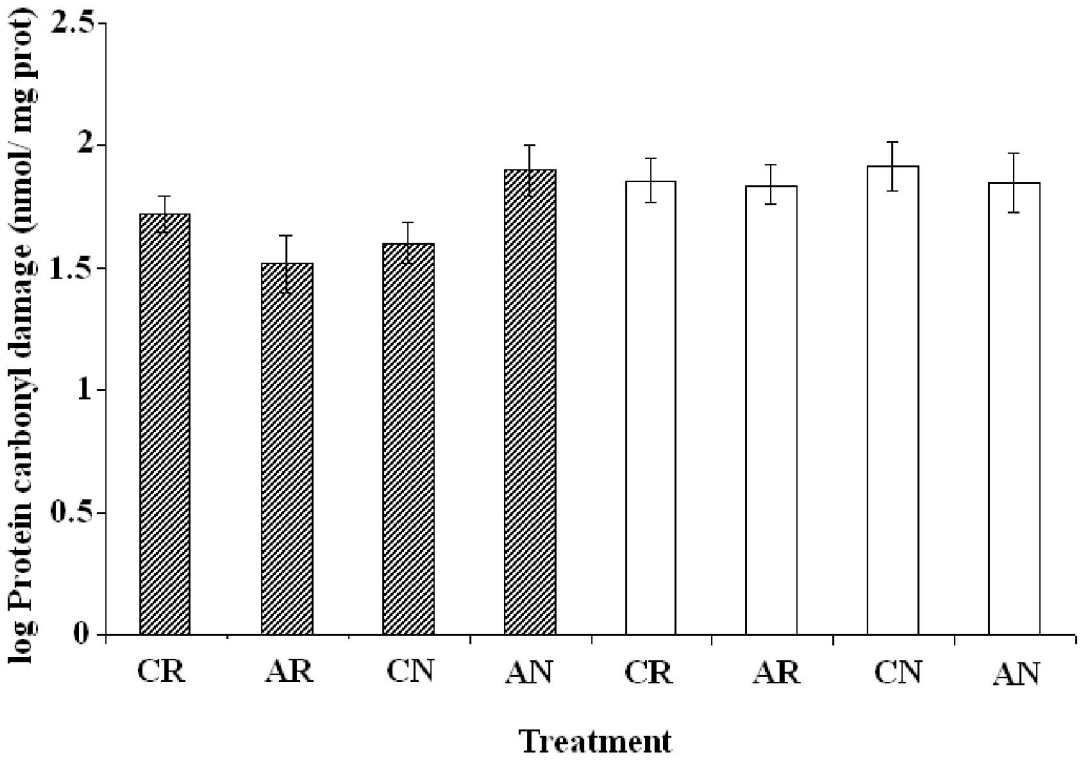


Figure 3.3 Mean ± s.e. protein carbonyls content (log transformed) in relation to feeding treatment group (hashed bars: high carotenoid diet, open bars: low carotenoid diet). Compensatory growth regime + resveratrol, *ad libitum* growth regime + resveratrol, compensatory growth regime without resveratrol, and *ad libitum* growth regime without resveratrol are denoted CR, AR, CN and AN, respectively; n = 118 fish.

Table 3.3 Results of a general linear mixed model examining protein carbonyl content in relation to growth regime, carotenoid supplementation, resveratrol supplementation and sex. Tank was included as a random factor. Non-significant variables were sequentially dropped from the analysis.

Final model	SE	DF	*t*	*p*
Carotenoid (L)	0.176	109	2.695	0.008
Growth regime (C)	0.178	106	0.077	0.939
Resveratrol (Y)	0.178	107	-0.678	0.499
Sex (M)	0.175	108	-1.580	0.177

3.4.3 Superoxide dismutase activity

In the analysis of SOD activity, a significant interaction was found between growth regime and whether fish were fed a diet supplemented with resveratrol or not (GLMM, t_{105} = 3.046, p = 0.003) (Table 3.4). *Ad libitum* growth regime fish that received resveratrol had significantly lower SOD activity (mean ± s.e. = 0.28 ± 0.02) than fish that did not receive resveratrol (mean ± s.e. = 0.41 ± 0.03, t-test, t = 3.409, p =0.001) (Figure 3.4). However, fish that received resveratrol had significantly higher SOD activity (mean ± s.e. = 0.44 ± 0.06) than fish that did not receive resveratrol (mean ± s.e. = 0.36 ± 0.04, t-test, t = -1.186, p = 0.024) (Figure 3.4).

A significant interaction was also found between growth regime and whether fish were fed a diet high or low in carotenoids (GLMM, t_{105}= 2.121, p = 0.036) (Table 3.4). Compensatory growth regime fish that received a high carotenoid diet had significantly lower SOD activity (mean ± s.e. = 0.33 ± 0.03) than fish that received a low carotenoid diet (mean ± s.e. = 0.46 ± 0.05, t-test, t = 1.97, p = 0.05). However, there were no differences in SOD activity within the *ad libitum* growth regime in relation to high (mean ± s.e. = 0.35 ± 0.03) or low carotenoid levels in the diet (mean ± s.e. = 0.33 ± 0.03, t-test, t = -0.667, p = 0.51) (Figure 3.5).

Table 3.4 Results of a general linear mixed model examining SOD activity in relation to growth regime, carotenoid supplementation and resveratrol supplementation. Tank was included as random factor**.**

Final model	Estimate	SE	DF	*t*	*p*
Resveratrol (Y)	-0.393	0.122	105	-3.218	0.002
Carotenoid (L)	-0.117	0.121	105	-0.968	0.336
Regime (C)	-0.375	0.158	105	-2.379	0.019
Resveratrol (Y) × Regime (C)	0.553	0.182	105	3.046	0.003
Carotenoid (L) × Regime (C)	0.382	0.180	105	2.121	0.036
Sex	-0.003	0.096	104	-0.035	0.972

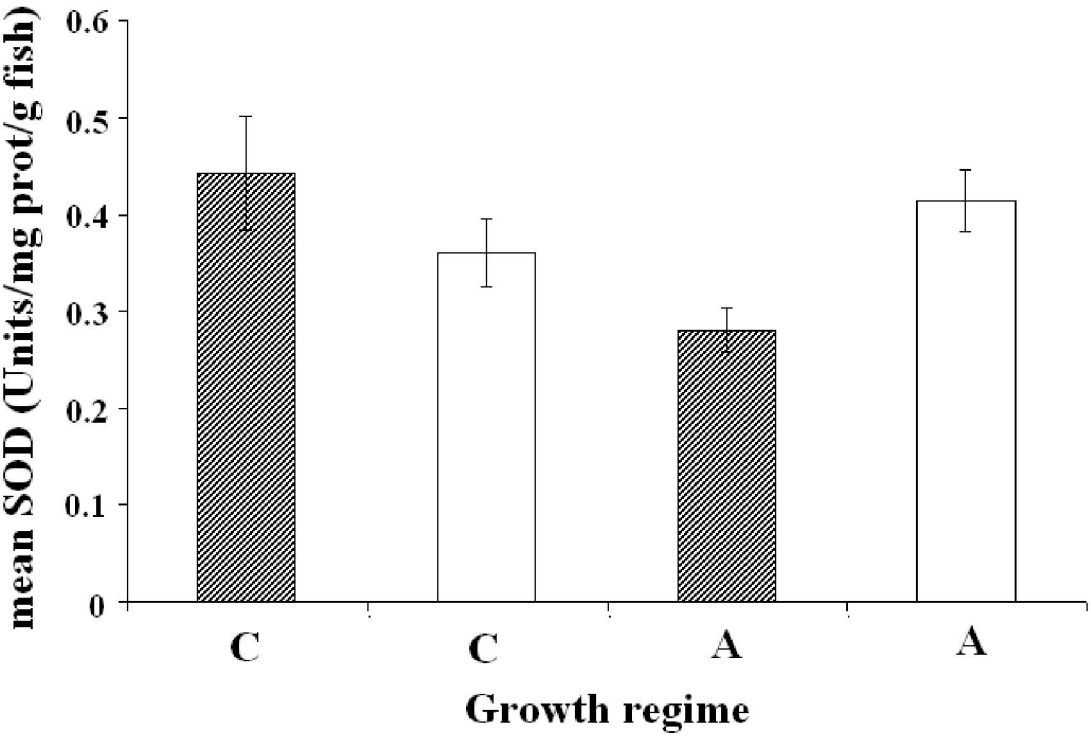


Figure 3.4 Mean ± s.e. SOD activity for fish fed either a diet with resveratrol (hashed bars) or without resveratrol (open bars) under a compensatory growth regime (C) or an *ad libitum* growth regime (A); n = 118 fish. Carotenoid supplementation groups have been combined in this figure to illustrate the effect of growth regime and resveratrol supplementation only.

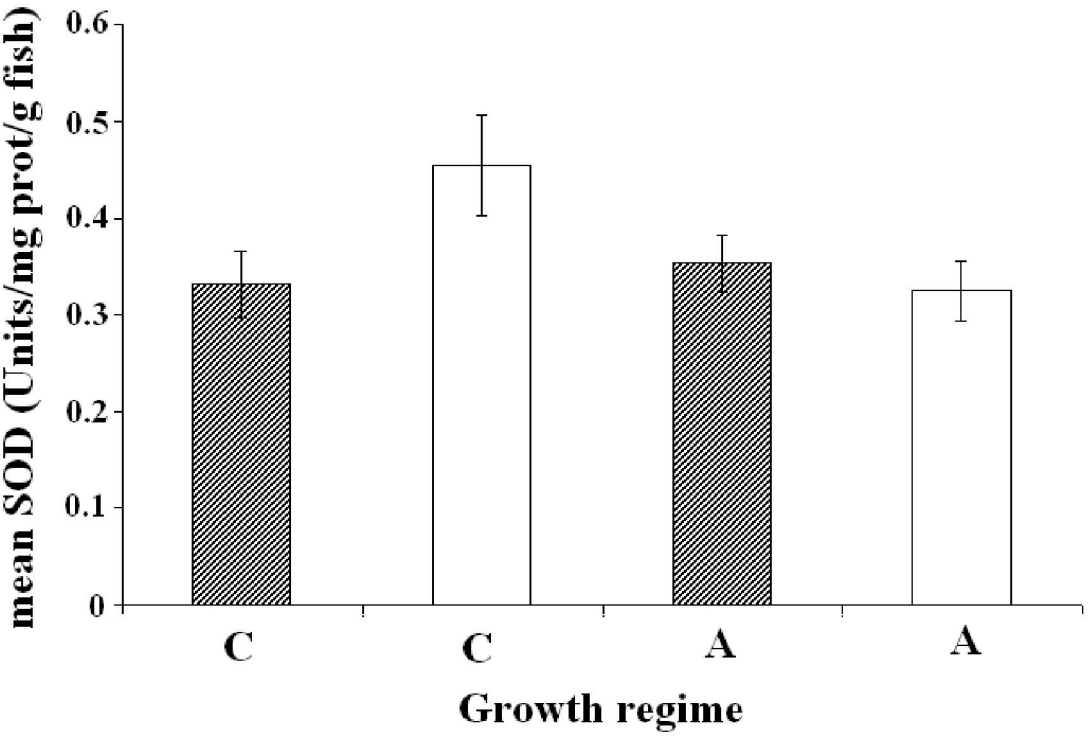


Figure 3.5 Mean ± s.e. SOD activity for fish fed either a high carotenoid (hashed bars) or low carotenoid (open bars) diet under a compensatory growth regime (C) or an *ad libitum* growth regime (A); n = 118 fish. Resveratrol supplementation groups have been combined to illustrate the effect of growth regime and carotenoid supplementation only.

3.4.4 Glutathione peroxidase activity

A significant interaction was found in the analysis of GPx activity between growth regime and whether fish were fed a diet supplemented with resveratrol or not (GLMM, t_{104} = 4.446, $p < 0.0001$) (Table 3.5). *Ad libitum* growth regime fish that received resveratrol had significantly lower GPx activity (mean ± s.e. = 0.033 ± 0.003) than fish that did not receive resveratrol (mean ± s.e. = 0.058 ± 0.005, t-test, t = 4.603, $p < 0.001$). However, there were no differences in GPx activity within the compensatory growth regime in relation to whether resveratrol was present (mean ± s.e. = 0.055 ± 0.007) or absent in the diet (mean ± s.e. = 0.039 ± 0.004, t-test, t = -1.979, p = 0.06) (Figure 3.6).

A significant interaction was also found between growth regime and whether fish were fed a diet high or low in carotenoids (GLMM, t_{104} = 2.108, p = 0.037) (Table 3.5). Compensatory growth regime fish that received a high carotenoid diet had significantly lower GPx activity (mean ± s.e. = 0.038 ± 0.004) than fish that received a low carotenoid diet (mean ± s.e. = 0.053 ± 0.006, t-test, t = 2.127, p = 0.04). However, there were no differences in GPx activity within the *ad libitum* growth regime in relation to high (mean ±

s.e. = 0.044 ± 0.004) or low carotenoid levels in the diet (mean ± s.e. = 0.043 ± 0.005, t-test, t = -0.194, p = 0.85) (Figure 3.7).

Table 3.5 Results of a general linear mixed model examining GPx activity in relation to growth regime, carotenoid supplementation and resveratrol supplementation. Tank was included as random factor.

Final model	Estimate	SE	DF	*t*	*p*
Resveratrol (Y)	-0.559	0.125	104	-4.475	<0.0001
Carotenoid (L)	-0.086	0.124	104	-0.697	0.488
Regime (C)	-0.602	0.162	104	-3.715	<0.001
Resveratrol (Y) × Regime (C)	0.834	0.188	104	4.446	<0.0001
Carotenoid (L) × Regime (C)	0.391	0.186	104	2.108	0.037

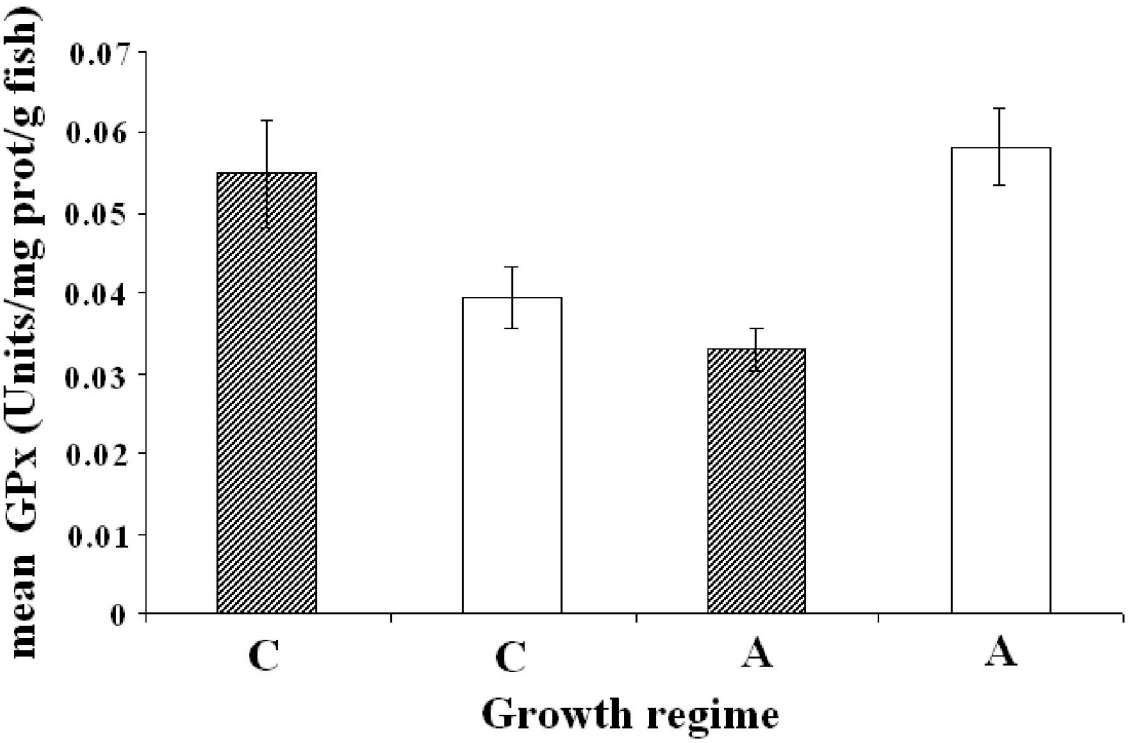


Figure 3.6 Mean ± s.e. GPx activity for fish fed either a diet with resveratrol (hashed bars) or without resveratrol (open bars) under a compensatory growth regime (C) or an *ad libitum* growth regime (A); n = 117 fish. Carotenoid supplementation groups have been combined to illustrate the effect of growth regime and resveratrol supplementation only.

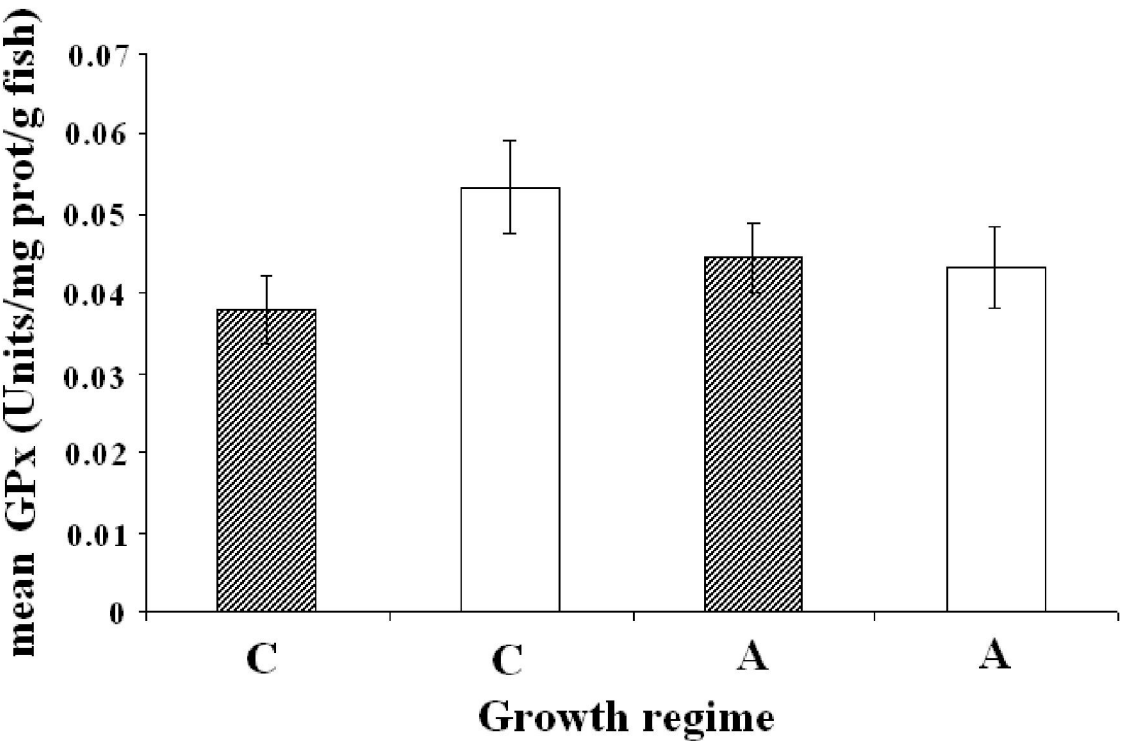


Figure 3.7 Mean ± s.e. GPx activity for fish fed either a high carotenoid (hashed bars) or low carotenoid (open bars) diet under a compensatory growth regime (C) or an *ad libitum* growth regime (A); n = 117 fish. Resveratrol supplementation groups have been combined to illustrate the effect of growth regime and carotenoid supplementation only.

3.4.5 Relationships between oxidative stress measurements

GPx activity was found to be correlated with protein carbonyl content (GLMM, t_{109} = 2.985, p = 0.031; Table 3.6): fish with high protein carbonyl content had higher levels of GPx activity (Figure 3.8). GPx activity was also found to be highly correlated with SOD activity (GLMM, t_{109} = 15.397, $p < 0.0001$; Table 3.6): fish with high SOD activity had higher levels of GPx activity (Figure 3.9).

Table 3.6 Results of a general linear mixed model examining GPx activity in relation to SOD activity and protein carbonyl content. Tank was included as random factor.

Final model	Estimate	SE	DF	*t*	*p*
Protein carbonyls	0.090	0.030	109	2.985	0.031
SOD	0.871	0.056	109	15.397	<0.0001

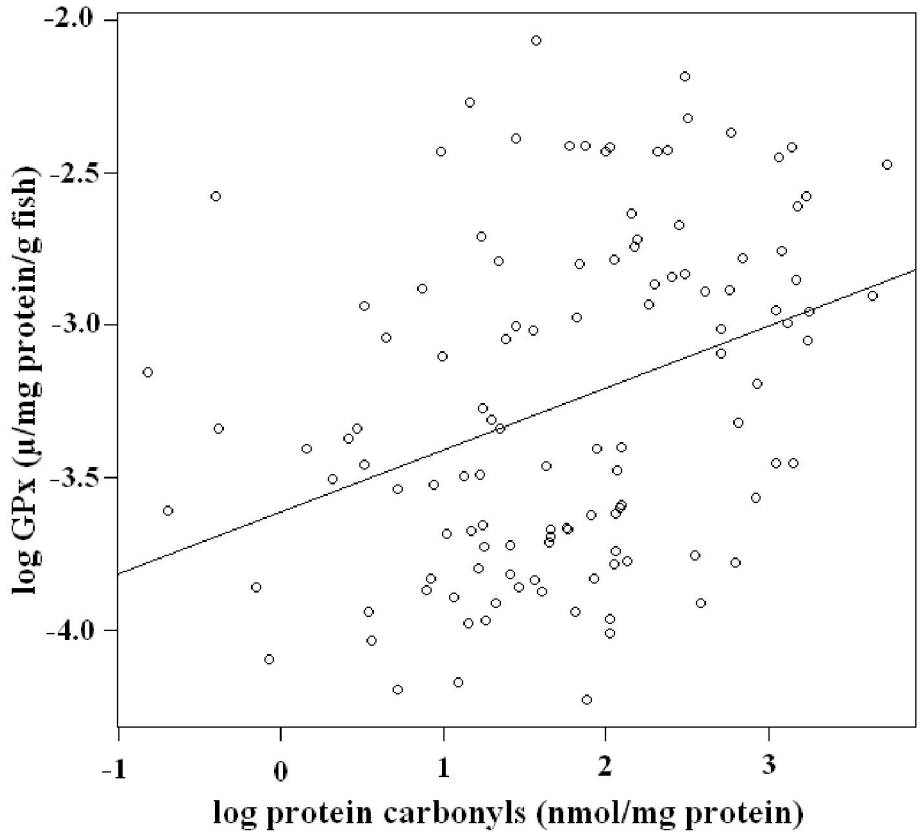


Figure 3.8 Relationship between the GPx activity and protein carbonyl content obtained for each individual; n = 117 fish. The linear regression line shown here is for illustrative purposes only (see text for full statistical analyses).

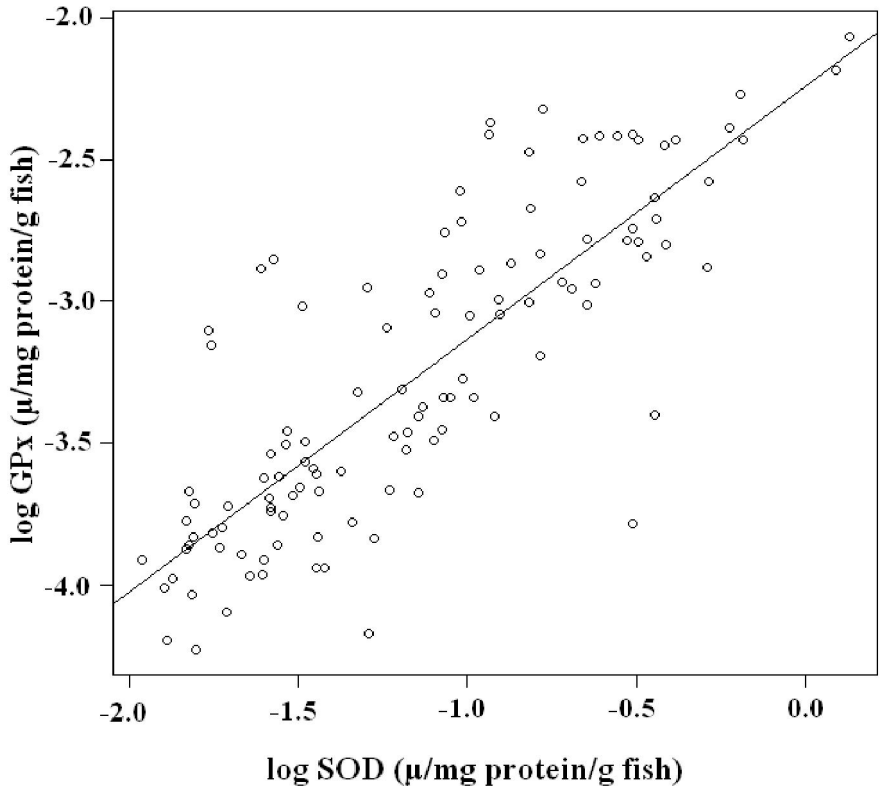


Figure 3.9 Relationship between the GPx activity and SOD activity obtained for each individual; n = 117 fish. The linear regression line shown here is for illustrative purposes only (see text for full statistical analyses).

3.4.6 Clutch mass

A significant interaction was found between the level of carotenoids in the diet and whether fish were fed a diet supplemented with resveratrol or not (GLMM, $t_{58} = 2.384$, $p = 0.020$) (Table 3.7). High carotenoid fish that did not receive resveratrol (mean ± s.e. = 0.011 ± 0.003) had significantly larger clutch masses than fish that had received resveratrol (mean ± s.e. = 0.005 ± 0.001, t-test, $t = 2.25$, $p = 0.032$) (Figure 3.10). However, there were no differences in clutch mass within the low carotenoid females in relation to whether resveratrol was present (mean ± s.e. = 0.008 ± 0.002) or absent in the diet (mean ± s.e. = 0.005 ± 0.001, t-test, $t = -1.084$, $p = 0.30$) (Figure 3.10). Clutch mass was found to be positively related to maternal length at the time of stripping (GLMM, $t_9 = 2.431$, $p = 0.037$; Table 3.7; Figure 3.11). Maternal oxidative stress status in terms of GPx activity, SOD activity and protein carbonyl content had no significant effect on clutch mass (Table 3.8).

Table 3.7 Results of a general linear mixed model examining egg clutch mass in relation to growth regime, carotenoid supplementation and resveratrol supplementation. The standard length of the fish at the time of stripping the females, the post-stripped fish mass and maternal oxidative stress in terms of GPx, SOD and protein carbonyl content were included as covariates. Tank was included as random factor.

Final model	Estimate	SE	DF	*t*	*p*
Resveratrol (Y)	-0.516	0.223	58	-2.313	0.024
Carotenoid (L)	-0.531	0.214	58	-2.482	0.016
Fish length	2.696	1.109	9	2.431	0.037
Carotenoid (Y) × Resveratrol (L)	0.802	0.337	58	2.384	0.020

Table 3.8 Results of a general linear mixed model examining egg clutch mass in relation to maternal oxidative stress status in terms of GPx, SOD and protein carbonyl content. Tank was included as random factor.

Final model	Value	SE	DF	*t*	*p*
SOD	-0.644	0.448	3	-1.463	0.246
GPx	0.485	0.409	3	-1.186	0.321
Protein carbonyl content	0.052	0.125	3	0.416	0.708

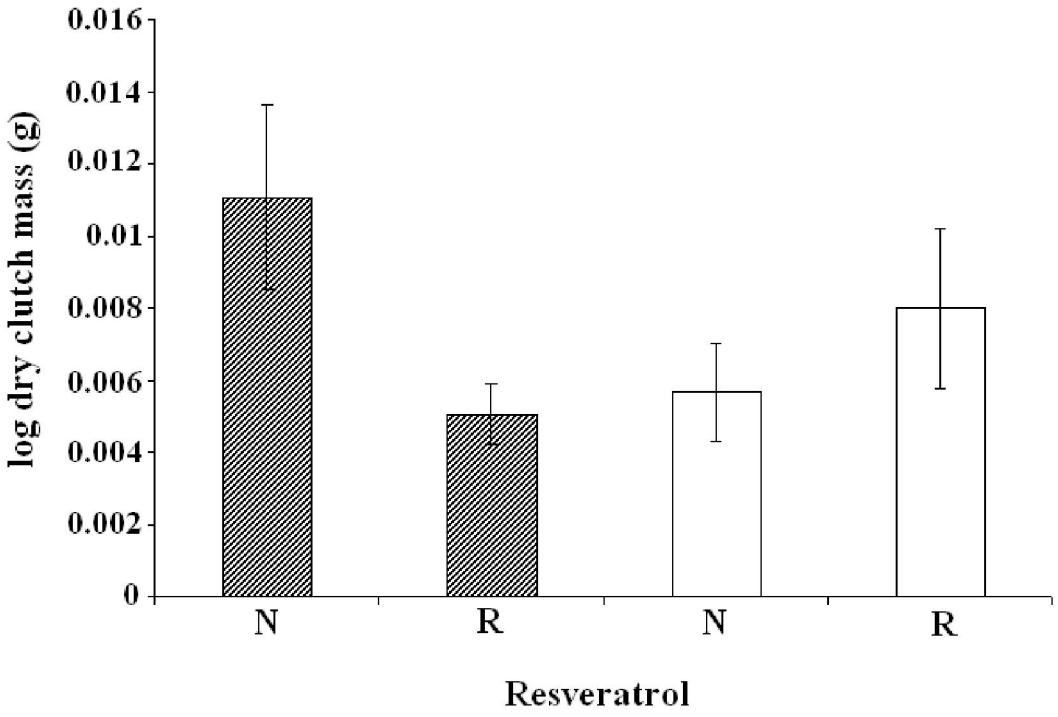


Figure 3.10 Mean ± s.e. dry clutch mass for mothers fed either a high carotenoid (hashed bars) or low carotenoid (open bars) diet with resveratrol (R) or without resveratrol (N). Growth regime groups have been combined to illustrate the effect of resveratrol and carotenoid supplementation only; n=71 clutches.

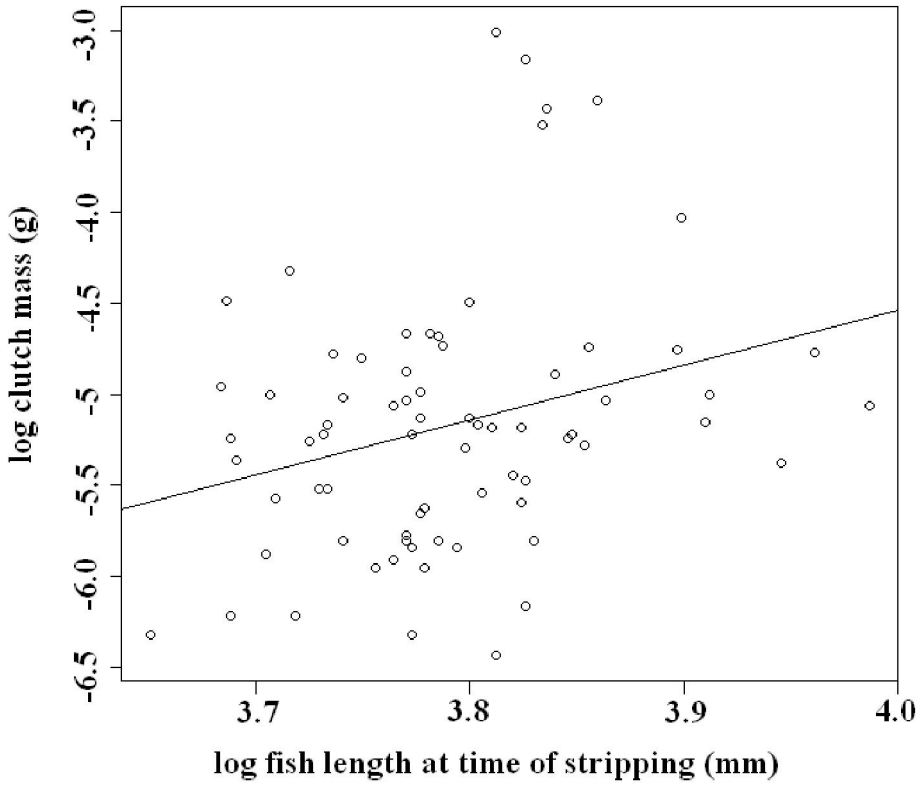


Figure 3.11 Relationship between mothers length at time of stripping and her egg clutch mass (g); n = 71 mothers. The linear regression line shown here is for illustrative purposes only (see text for full statistical analyses).

3.4.7 Antioxidant capacity in eggs and females

The OXY value of a female was not correlated with the OXY value obtained for her respective clutch (GLM: $F_{1,69} = 0.018$, $p = 0.893$; Figure 3.12). There were no significant effects of growth regime, carotenoid supplementation or resveratrol supplementation in the diet on the OXY values obtained for either the female sticklebacks (Table 3.10, Figure 3.13) or the clutches (Table 3.9, Figure 3.13).

Table 3.9 Results of a general linear mixed model examining egg OXY value in relation to growth regime, carotenoid supplementation and resveratrol supplementation. Tank was included as random factor.

Final model	Value	SE	DF	*t*	*p*
Resveratrol (Y)	-0.036	0.121	58	-0.296	0.77
Carotenoid (L)	0.035	0.116	58	0.300	0.77
Growth regime (C)	-0.137	0.121	58	-1.134	0.26

Table 3.10 Results of a general linear mixed model examining female OXY value in relation to growth regime, carotenoid supplementation and resveratrol supplementation. Tank was included as random factor.

Final model	Value	SE	DF	*t*	*p*
Resveratrol (Y)	-0.151	0.163	58	-0.926	0.36
Carotenoid (L)	0.245	0.159	58	1.539	0.13
Growth regime (C)	-0.152	0.163	58	-0.934	0.35

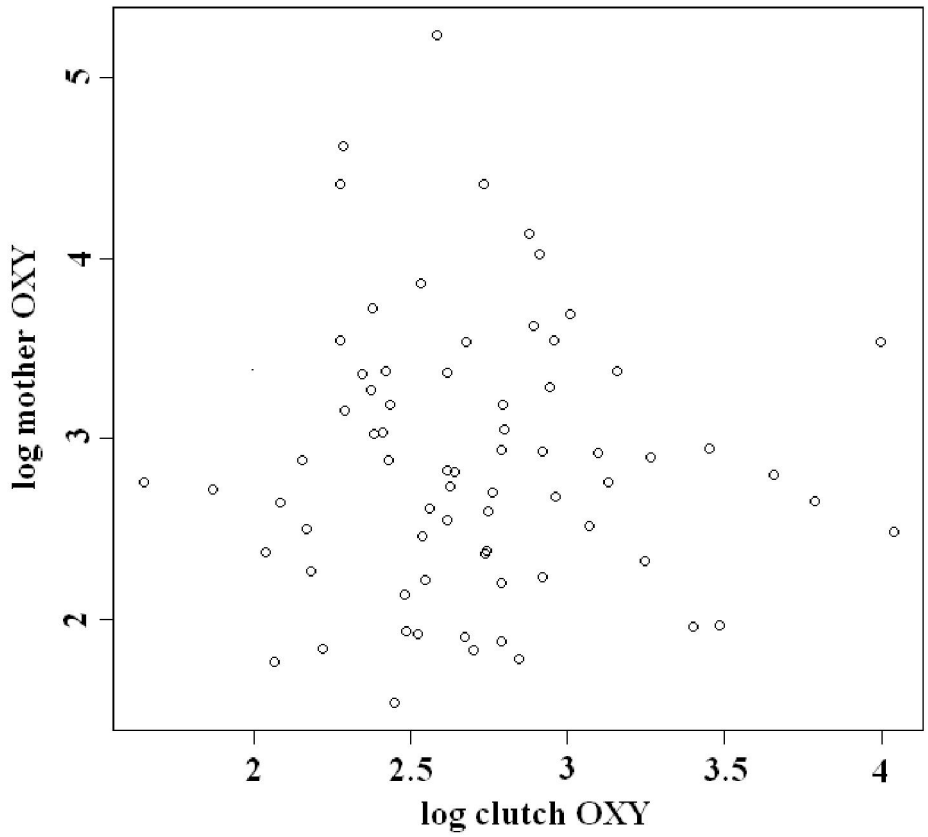


Figure 3.12 No relationship was identified between non-enzymatic antioxidant capacity in the mother and in her stripped clutch (OXY; in µmol mg protein/g^{-1} fish or egg of neutralised HOCl); n = 71 mothers and n= 71 clutches.

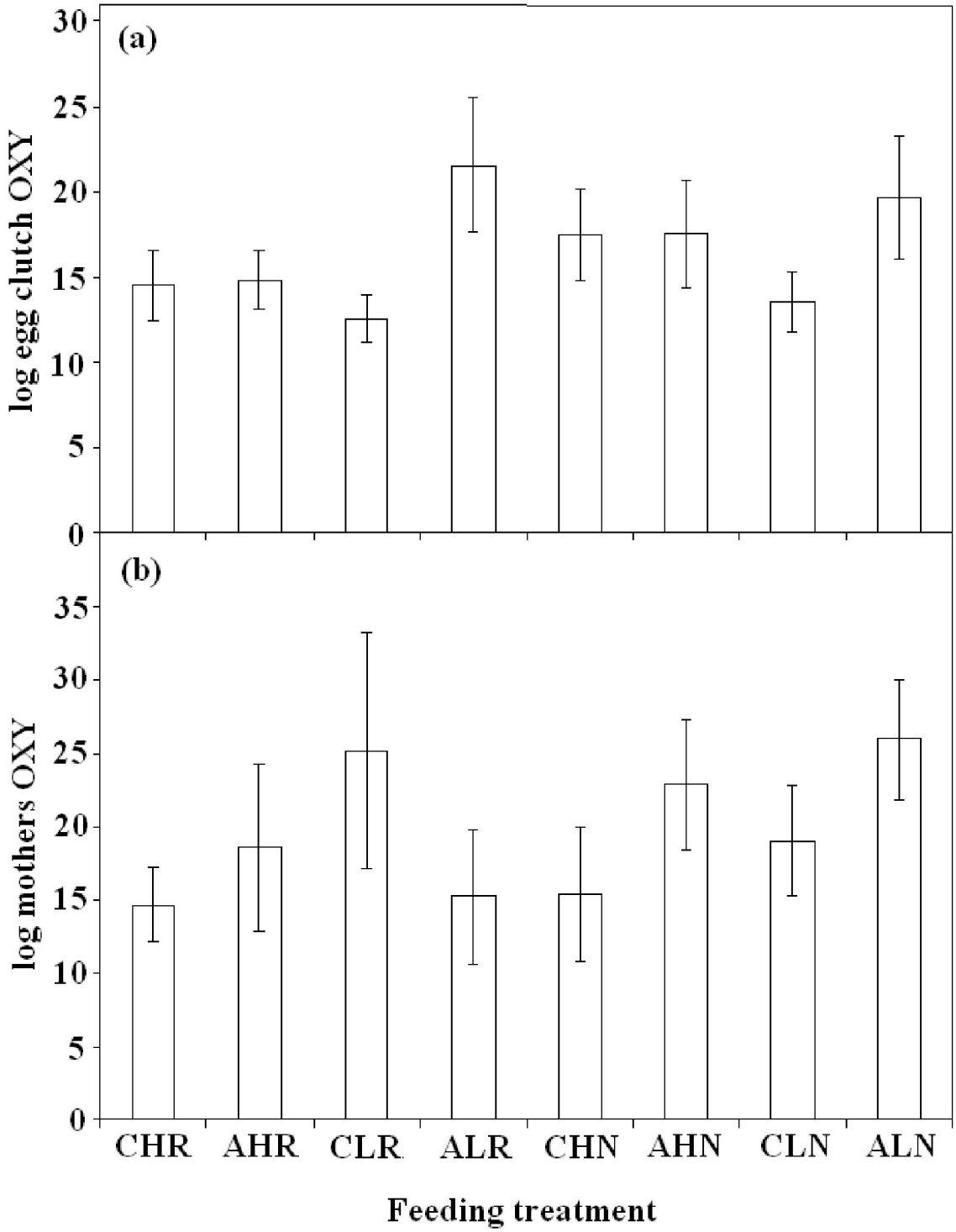


Figure 3.13 Mean ± s.e. non-enzymatic antioxidant capacity of a) clutches and b) mothers (log transformed OXY; in µmol mg protein/ g^{-1} fish or egg of neutralised HOCl) in relation to feeding treatment group. High carotenoid + resveratrol compensatory growth, high carotenoid + resveratrol *ad libitum* growth, low carotenoid + resveratrol compensatory growth, low carotenoid + resveratrol *ad libitum* growth, high carotenoid no resveratrol compensatory growth, high carotenoid no resveratrol *ad libitum* growth, low carotenoid no resveratrol compensatory growth and low carotenoid no resveratrol *ad libitum* growth denoted CHR, AHR, CLR, ALR, CHN, AHN, CLN and ALN respectively; n=71 mothers and n=71 clutches.

3.5 DISCUSSION

3.5.1 Compensatory growth response and female breeding performance

The degree to which growth compensation can occur after an environmentally induced growth depression differs widely between animal species but can also diverge within populations of the same species depending on their environmental conditions (Fraser et al. 2007). To achieve compensatory growth, an animal must reallocate its finite resources away from important traits and essential functions and instead redirect these resources towards expensive investments required for accelerated growth, and often increase their foraging effort (Dmitriew 2011). An absence of growth compensation occurs in animals in circumstances where the advantages of reaching a large adult body size are outmatched by the direct costs that would be incurred to the animal's fitness. For example, a dynamic optimisation model incorporating important elements of resource allocation has predicted that animals will not prioritise their energy budgets towards compensatory growth if there are large enough fitness benefits of investing this energy budget elsewhere, such as into sexual ornamentation (Lindström et al. 2005). In accordance with previous work on sticklebacks and the results of the previous experiment given in Chapter 2, the present study found that dietary restriction in early life resulted in a compensatory growth response after the food restriction had been uplifted (Zhu et al. 2003; Inness & Metcalfe 2008; Lee et al. 2010). A 3-week period of food restriction affected the growth trajectory of the fish during the food restriction period itself but also subsequently up until six weeks after this food restriction had been uplifted. This deviance in growth trajectories during the food restriction was buffered by the subsequent compensation period in terms of both length and mass. Therefore, the C growth regime fish fully compensated in terms of both energy reserves and skeletal growth.

In the present study, full growth compensation was achieved by the onset of the breeding season. Across many species, a large body size is positively correlated with many reproductive fitness benefits including higher fecundity, higher mating success and improved performance in intrasexual competition (Rowland 1989; Dickerson et al. 2002; Fokidis et al. 2007). This suggests that the sticklebacks may have invested their resources into achieving a larger body size in order to improve reproductive potential. Indeed, in the case of male three-spined sticklebacks, body size has been shown to be an important determinant of reproductive success in numerous studies (McLennan 2007; Kalbe et al.

2009; Sparkes et al. 2013), while a larger body size is associated with higher fecundity in females (Wootton 1973; Wootton 1979). These findings indicate that in the present study the reproductive fitness benefits of reaching a size threshold in time for the breeding season may have considerably outweighed the physiological costs associated with undergoing compensatory growth. However, more recently, it has come to light that reaching a large body size through compensatory growth can also be associated with costs to reproduction (Auer et al. 2010a; Lee et al. 2012).

Despite reproduction being a key component of fitness, the reproductive costs of compensatory growth are only beginning to be realised due to a past predominant focus of its effects on survival (Johnsson & Bohlin 2006; Dmitriew & Rowe 2007). For instance, reproductive costs following juvenile compensatory growth have been demonstrated in Trinidadian guppies *Poecilia reticulata*, where compensatory growth resulted in a 20% decline in offspring production (Auer et al. 2010a). However, in the present study a compensatory growth regime had no influence on female clutch mass. This suggests that the females may have been able to fully repair any damaging effects incurred during the period of compensatory growth by the time the breeding season had commenced. Alternatively, it could be the case that the costs of compensatory growth did not affect egg production but may have been costly to other phenotypic traits not related to fecundity, such as survival. In hindsight, it would have been interesting to determine whether the females that had exhibited compensatory growth would have survived to their second breeding season and if so, still achieved comparable clutch sizes to their continuous growth peers. Nevertheless, the present study found that high carotenoid fish that did not receive resveratrol had significantly larger clutch masses than high carotenoid fish that had received resveratrol. This suggests that resveratrol may have imposed a detrimental effect on egg production. Indeed, this seems plausible as resveratrol is not naturally found in the diet of three-spined sticklebacks. It is possible that there was a mild toxic effect of resveratrol on egg production which may have potentially caused an increase in free radical production. Xenobiotic metabolism has been suggested to occur in mice supplemented with lifelong α-tocopherol, where an absence of a significant effect of its supplementation on oxidative damage has questioned its antioxidant properties (Selman et al. 2008). Additionally, α-tocopherol and ascorbic acid supplementation in short-tailed field voles *Microtus agrestis* lacked to have a positive effect on DNA oxidative damage to hepatocytes and lymphocytes and in fact reduced lifespan (Selman et al. 2013). This study by Selman et al. (2013) highlights the growing evidence that there is significant variation

between species in terms of the effects dietary antioxidants have in influencing oxidative stress and lifespan. A microarray analysis found that after two months of α-tocopherol supplementation, specific genes, linked to its degradation were upregulated as much as 3-fold in comparison with controls in the livers of female mice. Therefore, it was likely that α-tocopherol underwent detoxification (Selman et al. 2008). As resveratrol did not reduce oxidative damage to proteins in the present study, perhaps xenobiotic metabolism may have occurred and therefore nullified any potential antioxidant effects of resveratrol. In fact, resveratrol has been previously shown to alter xenobiotic metabolising enzymes in the liver and lungs of male mice (Canistro et al. 2009). Mixed results were produced across phase I "bioactivating" and phase II "detoxifying" enzymes related to xenobiotic metabolism (Canistro et al. 2009). These results highlight the requirement for a greater understanding of resveratrol's toxicological profile. Resveratrol's limited efficacy *in vivo* may relate to our relatively poor understanding of its distribution, metabolism and excretion (Canistro et al. 2009).

Neither growth regime nor dietary supplementation of resveratrol and carotenoids had an effect on the non-enzymatic antioxidant capacity of the females or her eggs. The absence of an effect of carotenoid level in the diet is surprising as it was predicted that their presence would make egg production less physiologically demanding. It was expected that female non-enzymatic antioxidant capacity would be lower in low carotenoid females that had produced eggs as she would have been left with less to cope with other physiological functions such as scavenging reactive oxygen species (Costantini 2010). However, female non-enzymatic antioxidant capacity was measured considerably after spawning and therefore this may explain the lack of correlation between the non-enzymatic antioxidant capacity of the females and her eggs.

3.5.2 Oxidative stress status in the mature three-spined sticklebacks

Compensatory growth and breeding are energetically demanding activities which potentially exposed the fish to physiological costs. One such cost may be that the fish would have had to cope with an increase in free radical production during catch-up growth and/or during the subsequent breeding season (Alonso-Alvarez et al. 2004; Alonso-Alvarez et al. 2007). In the present study the level of oxidative damage that they incurred was influenced by their relative intake of carotenoids, with a diet higher in carotenoids significantly reducing oxidative damage to proteins. However, the mechanism by which

the carotenoids may have inhibited protein carbonylation cannot be determined from this study. Protein carbonyl concentrations were not elevated in fish that experienced compensatory growth. However, these fish were culled 19 weeks after they had fully compensated in growth in order to assess breeding investment. Therefore, it is possible that the fish may have had time to remove the carbonylated proteins caused by compensatory growth by the time they were culled for analysis. This finding is interesting in itself since it suggests that the fish's antioxidant repair systems may have been able to remove and replace the damaged molecules, therefore eliminating the possibility of long term negative effects of compensatory growth. One of the main setbacks of using three-spined sticklebacks in the present study was that they could not be repeatedly sampled for oxidative stress as sampling involved sacrifice of the animal.

In the present study, the differences in endogenous antioxidant enzyme upregulation are complex, with interactions emerging separately between growth regime and the two different dietary supplements. For instance, within the compensatory growth regime, the fish that had been fed resveratrol had significantly increased their levels of SOD in comparison with the fish that had not been fed resveratrol, in order to achieve comparable levels of oxidative damage to proteins. This suggests that there may have been a negative effect of resveratrol supplementation on oxidative stress which increased the requirement for the endogenous antioxidant defence system to upregulate the production of SOD, an enzyme which is known to play a key role in the quenching of reactive oxygen species (McCord & Fridovich 1969; Finkel & Holbrook 2000). Resveratrol has also been previously shown to induce the expression of SOD in cardiomycocytes, *in vitro* (Cao & Li 2004). If resveratrol was indeed having a mild toxic pro-oxidant effect in the present study, then it is likely that an increased ROS production resulted in the greater provision of SOD. This is an interesting result, since in circumstances when resources are limiting, an increased upregulation of SOD would have come at a cost to investment elsewhere. As a consequence, other life history traits may have been compromised, as the upregulation of antioxidant defences may have had to be traded against other physiological functions, therefore affecting fitness in different ways. Indeed, ROS production has been proposed to be an important contributing factor that mediates numerous life history trade-offs associated with fitness-related traits such as reproduction and immunity (Dowling & Simmons 2009). For example, a trade-off between reproductive investment and oxidative protection was found in zebra finches *Taeniopygia guttata*, where parents were found to sacrifice oxidative protection for parental care when their brood size was experimentally

enlarged (Wiersma et al. 2004). In the larvae of the caddisfly *Limnephilus rhombicus*, those forced to build a new case elicited an increase in their antioxidant defences in order to achieve protein oxidative damage comparable with those that had not required to invest in rebuilding a new case (Mondy et al. 2012). However, a trade-off appeared to have occurred as a result of this as male body size was reduced in those that had to rebuild a new case. In addition, there was also a 28% reduction in the abdominal dry mass of the males produced from the larvae that had to reconstruct a new case which indicates that reproductive capacity could have also been reduced (Mondy et al. 2012).

The carotenoid content of the diet had a significant effect on the upregulation of endogenous SOD and GPx defences. Fish on a low carotenoid diet that had exhibited compensatory growth had higher levels of SOD and GPx than low carotenoid fed fish that had been fed *ad libitum*. The high carotenoid fish that had exhibited compensatory growth had comparable SOD and GPx activities to the high carotenoid *ad libitum* growth fish. This suggests that the high dose of carotenoids worked as adequate dietary antioxidants to cope with the presumed greater production of ROS during the phase of compensatory growth. Similar findings have been described in Tocher et al. (2002), where positive dietary antioxidant effects of vitamin E were found in three commercially important marine fish species, which as a result reduced oxidative damage and alleviated the requirement to upregulate endogenous antioxidant enzymes including SOD and GPx (Tocher et al. 2002).

3.5.3 Conclusions

The beneficial effects of carotenoids in the present study exemplify and make progressive advances in determining the importance of dietary antioxidants in mediating trade-offs. The results also suggest that dietary antioxidants such as resveratrol that are not present naturally in the diet of study species might in fact promote the production of ROS. The present study also highlights the antioxidant function of carotenoids in three-spined sticklebacks. This result suggests that although carotenoids appear to be unimportant antioxidants for birds (Costantini & Møller 2008; Perez-Rodriguez 2009), these findings should not be generalised across all taxa.

CHAPTER 4 – THE RELATIVE ROLES OF DIETARY CAROTENOIDS AND RESVERATROL IN THE TRADE-OFF BETWEEN MALE REPRODUCTIVE INVESTMENT AND OXIDATIVE STRESS

4.1 ABSTRACT

There is growing evidence to suggest that compensatory growth may have deferred costs on reproductive traits such as breeding behaviour, breeding colouration and sexual attractiveness. To our knowledge, this is the first study to investigate the combined dietary effects of carotenoids (two non-vitamin lipophilic compounds) and resveratrol (a non-vitamin hydrophilic compound) on mediating the expression of a carotenoid-based sexual ornament after a period of compensatory growth. The results of this study suggest that the male three-spined sticklebacks that were provided with a limited supply of dietary carotenoids continued to disproportionately invest a plentiful supply of carotenoids into their sexual signal at the expense of their body carotenoids, despite these body carotenoids being presumed to play vital roles in other important physiological functions such as in the detoxification system. Therefore, distributing their limited carotenoid resource in this manner was predicted to increase their levels of oxidative stress. Indeed, the male three-spined sticklebacks fed a low carotenoid diet did have higher oxidative damage in terms of protein carbonyl content at the end of the breeding season. The relatively large investment of their sexual signal at the beginning of the breeding season appears to have come at a cost towards the end of the season as indicated by their higher oxidative damage levels to proteins. Resveratrol did not play a role in mediating the expression of the male's sexual ornament in the sticklebacks. Interesting correlations were found between aspects of nest building performance, red throat intensity and oxidative stress status. For instance, males that produced redder throats during the breeding season had lower levels of protein carbonyl content and lower GPx activity at the end of the breeding season. Overall, these results clearly demonstrate that during the breeding season, nutritional circumstances, even weeks prior to the breeding season, can strongly determine how an individual balances investment in the breeding attempt alongside investment in self maintenance such as combating the deleterious effects of oxidative stress associated with compensatory growth.

4.2 INTRODUCTION

4.2.1 Compensatory growth, reproductive investment and oxidative stress

It has long been recognised that compensatory growth could be beneficial for reproduction through its positive effects on size and age at maturity (Ali et al. 2003). However, it has now been recognised that compensatory growth may have deferred costs on reproductive traits such as breeding behaviour, breeding colouration and sexual attractiveness (Álvarez 2011; Lee et al. 2012), although this area of research is still relatively unchartered and the minimal evidence available in the literature to date is mixed (Dmitriew 2011). For instance, juvenile growth compensation in Trinidadian guppies *Poecilia reticulata* resulted in a 20% decline in offspring production (Auer et al. 2010a). After exhibiting full growth compensation prior to the breeding season, male three-spined sticklebacks displayed reduced sexual ornamentation and demonstrated slower nest building rates than their peers who had experienced unrestricted growth (Lee et al. 2012). Moreover, clutch investment (in terms of clutch size, egg size, and number of eggs produced per year) was also reduced in female three-spined sticklebacks that had exhibited compensatory growth (Lee et al. 2012). However, such negative effects are not universal: in the ladybird beetle *Harmonia axyridis*, compensatory growth had no effect on female fecundity, male attractiveness or male mating behaviour (Dmitriew & Rowe 2007). Interestingly, although compensatory growth has been found to have no direct effect on sexual attractiveness in green swordtails *Xiphophorus helleri* (Walling et al. 2007), it has been proposed that its known negative effects on dominance and swimming performance may have the capacity to indirectly reduce reproductive performance (Royle et al. 2005; Royle et al. 2006; Álvarez 2011).

As described in Chapter 3, there is growing evidence showing that compensatory growth can result in an increased susceptibility to oxidative stress (De Block & Stoks 2008; Furné et al. 2009; Larcombe et al. 2010a; Bayir et al. 2011), and this may be the mechanism through which compensatory growth results in costs to reproduction (De Block & Stoks 2008). Reproduction itself has also been associated with oxidative stress and it has been proposed that there is duality in the direction of its causality (Losdat et al. 2011; Stier et al. 2012). For instance, although reproduction has been associated with an increase in oxidative damage post-breeding, it has also been found that an increase in oxidative stress can reduce reproductive potential prior to breeding (Stier et al. 2012). Therefore, oxidative

stress can constrain reproduction but can also come at a cost of reproduction (Costantini 2008).

Focus in this research area began in fruit flies *Drosophila melanogaster*, where a manipulated increase in female egg production was associated with an increased susceptibility to oxidative stress (Salmon et al. 2001; Wang et al. 2001). However susceptibility to oxidative stress was only crudely measured as survival against exposure to a toxic substance (Paraquat) (Salmon et al. 2001; Wang et al. 2001). Most of the progress in this research area has focused solely on measuring the relationship between reproductive effort and antioxidant defences (Wiersma et al. 2004; Bize et al. 2008). For example, antioxidant activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were found to decrease with brood size in zebra finches *Taeniopygia guttata,* suggesting that an increase in reproductive investment results in a depletion of antioxidant defences (Wiersma et al. 2004). This has also been supported in a study on great tits *Parus major*, where a larger brood size induced a decrease in antioxidant capacity (Losdat et al. 2011). In contrast, antioxidant defences were found to increase with clutch size in alpine swifts *Apus melba* which suggests that an increase in reproductive investment results in an upregulation of antioxidant defences (Bize et al. 2008). However, the results of these earlier studies are quite difficult to interpret as measuring antioxidant defences alone is deemed an inadequate method of demonstrating the level of oxidative stress incurred by an animal, and it does not reflect their levels of oxidative damage either (Costantini & Verhulst 2010). Recently, this issue has become more recognised and a wider range of assays are being used to provide a more comprehensive assessment of oxidative stress status (Alonso-Alvarez et al. 2010; Beaulieu et al. 2011). For example, a recent study measured both antioxidant capacity and a biomarker of oxidative damage (lipid peroxidation) and found that an increase in hatching effort caused an increase in oxidative damage in female red-legged partridges *Alectoris rufa* (Alonso-Alvarez et al. 2010). The females with higher hatching success suffered higher lipid peroxidation in their erythrocytes, but showed no differences in their total antioxidant capacity (Alonso-Alvarez et al. 2010).

However, alongside these developments, there have been a suite of recent studies investigating links between oxidative stress levels and reproduction which have provided inconclusive or negative results (Nussey et al. 2009; Garratt et al. 2010; Bergeron et al. 2011; Isaksson et al. 2011; Markó et al. 2011; Oldakowski et al. 2012). Most of these

studies highlight a limitation in experimental design which may have confounded the results; *ad libitum* resource availability may alleviate the effects of reproductive investment on oxidative damage. For example, no relationship was found between oxidative damage and female reproductive investment (both litter size and mass) in the spotted snow skink *Niveoscincus ocellatus* (Isaksson et al. 2011). However, these experimental animals received a plentiful supply of food and therefore this study - among others - neglects the issue that trade-offs will only exist when resources are limited (Van Noordwijk & de Jong 1986). As pointed out recently (Metcalfe & Monaghan 2013) this could explain the absence of significant co-variations found between measures of female reproductive investment and oxidative stress in many studies where food availability was either *ad libitum* or unaccounted for (in studies investigating free-living subjects) (Garratt et al. 2010; Beaulieu et al. 2011; Bergeron et al. 2011; Oldakowski et al. 2012). Therefore, the present study included a compensatory growth regime to increase susceptibility to oxidative stress but also to enable the investigation of the effects of a limited resource supply (in terms of quantity) in early life on reproduction and how different levels of dietary antioxidants may influence this.

4.2.2 Dietary antioxidants, sexual ornamentation and oxidative stress

Sexual traits are proposed to be indicative of a male's quality to females, since investment in sexual ornamentation may incur costs to other traits when resources are in short supply (Zahavi 1975). Carotenoid-based sexual signals have received considerable attention in this area of literature (Lindström et al. 2009; Svensson & Wong 2011; Skibsted 2012). When carotenoid availability is limited in the diet, this is suggested to result in a trade-off in their allocation within the body for both signal expression and in their alternative roles (Lozano 1994; Chew & Park 2004). Positive relationships have been found between a male's sexual signal investment and his performance in reproduction, immune defence and oxidative stress resistance, suggesting that the carotenoid-based signal can indeed be an honest advertisement of quality (Blount et al. 2003b; Peters et al. 2004; Pike et al. 2007a; Navara et al. 2012).

Oxidative stress has been suggested to be a potential underlying mechanism which mediates these trade-offs and allows honesty to be retained in carotenoid-based sexual signalling (von Schantz et al. 1999). However, more recently the antioxidant function of carotenoids has been highly debated in the literature (Perez-Rodriguez 2009). Although

stronger carotenoid-based sexual signals have been linked to reduced oxidative stress levels, there is little evidence to suggest that carotenoids themselves directly reduce oxidative stress (Costantini et al. 2006; Costantini et al. 2007b; Costantini & Møller 2008; Simons et al. 2012). In addition, it has been suggested that a plausible reasoning for links between oxidative stress levels and the strength of carotenoid-based sexual signals may be due to the sensitivity of carotenoids to levels of reactive oxygen species, which promote the oxidative decolouration of carotenoids (Hartley & Kennedy 2004). Therefore, it could be the quality of the antioxidant system comprised of alternative antioxidants which mediates the expression of carotenoid-based signals and not the carotenoids themselves (Hartley & Kennedy 2004).

In line with this theory, it has been previously shown that an increase in the availability of two non-carotenoid antioxidants (vitamins C and E) increased the expression of the carotenoid-based nuptial throat colouration in male three-spined sticklebacks and furthermore increased antioxidant capacity (Pike et al. 2007b). These findings are consistent with the hypothesis that the carotenoid-based signal advertises the availability of colourless antioxidants rather than solely reflecting the antioxidant capacity of carotenoids (Hartley & Kennedy 2004). However, these effects have not been found in more recent work in bird species (Karu et al. 2008; Orledge et al. 2012). For example, in ring-necked pheasants *Phasianus colchicus*, the availability of dietary vitamin E had no influence on sexual attractiveness in this strongly sexually selected species (Orledge et al. 2012). Neither was the carotenoid-based plumage colouration affected by dietary vitamin E content in male greenfinches *Carduelis chloris* (Karu et al. 2008).

It has become increasingly obvious from developments in this field that the links between dietary antioxidant intake (both carotenoids and colourless antioxidants), sexual signalling and oxidative stress levels are inherently complex (Garratt & Brooks 2012). The importance of investigating the effects of different antioxidant classes in combination with one another has been emphasised (Mortensen et al. 2001; Skibsted 2012). For instance, it has been highlighted that the combined role of non-vitamin lipophilic and hydrophilic compounds requires more attention, since the few natural hybrids of carotenoids and plant polyphenols which do exist in the wild have particularly efficient antioxidant capacities e.g. the xanthophyll Dimethoxyisorenieratene (DMIR) (Martin et al. 2009; Skibsted 2012).

The present study hypothesised that if resveratrol played a sufficient role as a dietary antioxidant, then males supplemented with resveratrol alongside a high dose of carotenoids were predicted to invest "spare" carotenoids in producing a stronger carotenoid-based sexual signal. In addition, positive effects of such a diet were hypothesised to be seen in terms of investment in nest building as male three-spined sticklebacks provided with a higher carotenoid availability have been found to exhibit a significantly better standard of parental care (Pike et al. 2007c). For instance, males fed a high carotenoid diet have shown higher clutch hatching success than males fed a low carotenoid diet (Pike et al. 2007c).

4.2.3 Aims

The present study implemented a compensatory growth regime and manipulated dietary carotenoid and resveratrol availability in three-spined sticklebacks from early life and quantified their effects on self maintenance (in terms of oxidative stress) in relation to the expression of the males' sexual signal investment during the breeding season and their performance in nest building. Therefore, it was also possible to investigate whether greater investment of resources in these reproductive traits came at a cost for self maintenance in males that had been fed a diet low in carotenoids and lacking in resveratrol. This was hypothesised to be reflected in an increased oxidative stress status in these males at the end of the breeding season. Therefore, the present study was able to determine whether dietary carotenoid and resveratrol availability played a beneficial role in regulating any such trade-offs.

4.3 METHODS

4.3.1 Source of fish and rearing conditions

The male three-spined sticklebacks from the experiment previously described in Chapter 3 were examined during the breeding season (late April until late July) (Figure 4.1). The breeding season commenced on 20th April 2011 when the first signs of male red nuptial throat colouration became apparent. As described in Chapter 3, the males were separated into individual tanks as soon as their sex could be identified due to their nuptial throat colouration. These individual (7-L) tanks were identical in size to their original group tanks (33 × 18 × 19 cm). From the onset of the breeding season (20th April 2011), each male was presented with a gravid female once a day for ten minutes, in order to prompt full

expression of his nuptial throat colouration and to also later stimulate nest building once nesting material had been provided (Pike et al. 2011). A full description of the timing of the growth measurements of the fish is given in Chapter 3.

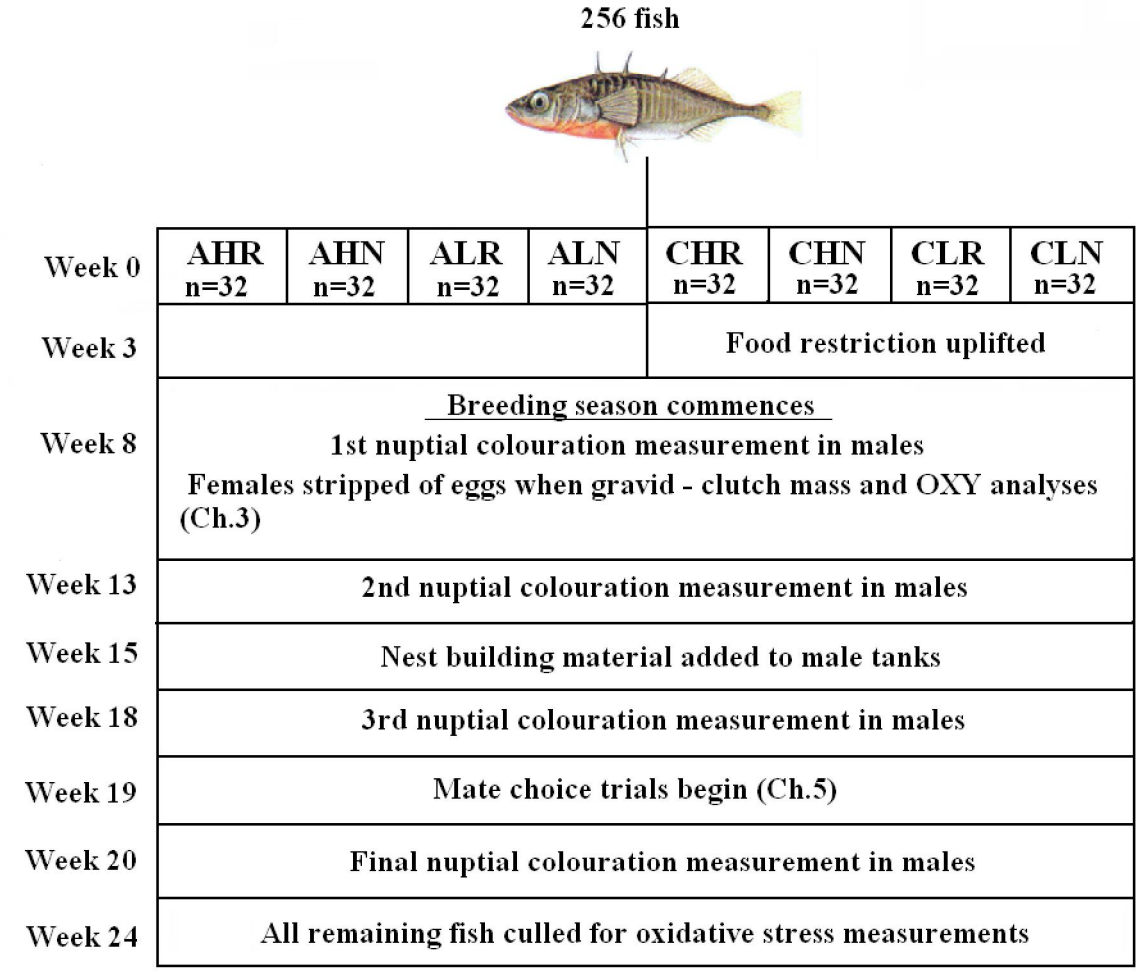


Figure 4.1 Schematic of experimental design. Fish were equally divided into the eight feeding treatments described in (Table 2.1, Chapter 2). At week 3 the food restriction was uplifted in the four compensatory growth regime fish (CHR, CHN, CLR and CLN) (See Chapter 3, Figure 3.2). At week 8, 13, 18 and 20, males were photographed to measure their nuptial throat intensity. At week 15, nesting material was introduced into the male tanks to assess nest building parameters. At week 24, all remaining fish were culled for oxidative stress measurements (See Chapter 3).

4.3.2 Measurement of nest building

Nesting material was introduced into all male tanks on the 10th June 2011 prior to the males commencing their first breeding round. This nesting material consisted of a nesting dish (a Petri dish filled with fine sand) and building material (one hundred 5cm long black polyester threads). Black polyester threads have been successfully used in previous three-spined stickleback studies to mimic naturally available nest building materials e.g.

(Rushbrook et al. 2010; Lee et al. 2012). Two aspects of nest building were recorded based on previous work carried out by Barber et al. (2001): the number of days it took the males to begin constructing the nest after being supplied with nesting material and the total number of days it took the males to complete the nest after being supplied with the nesting material. A nest was regarded as complete when a nest entrance was visible (Rushbrook & Barber 2008; Rushbrook et al. 2010). The status of each tank was checked daily to assess these two aspects of nest building, and they were measured using a similar scoring system adopted in Lee et al. (2012). Nest building rate was scored from 8 (nest completed on 1st day that material was supplied) to 1 (nest completed on 8th day) and the males obtained a score of 0 if they had still not completed nest building by the end of the 8th day. Pre-building lag was also scored from 8 (the male started to manipulate nesting material on 1st day) to 1 (started to manipulate nesting material on 8th day). The assessment of nest building rate commenced immediately after the building material was supplied and therefore included the "pre-building lag" time. There were no males that had not begun manipulating the nesting material by the 8th day and therefore no males obtained a score of 0 for pre-building lag rate. Following the removal of the males from their nesting aquaria on their final growth measurement, the nests were then carefully removed from their sandy substratum and collected on separate Petri dishes which were labelled according to fish identity and dried for 8 hours in a drying oven. The dried threads were then separated out from any remaining sandy substratum and weighed to the nearest 0.001g. An accurate estimation of the total number of threads used to build the nest was then calculated by dividing the dry weight of the nesting material by the dry weight of one 5cm long black polyester thread.

4.3.3 Measurement of nuptial colouration

The males were photographed at four time points throughout the breeding season in order to measure the intensity of their red nuptial throat colouration. Time point 1 (20th April 2011) was when the males had first started to develop red nuptial throat colouration. The three subsequent measurements (30th May, 10th July and 27th July 2011) were carried out during the breeding season alongside their growth measurements which are described in Chapter 3. These four time points were all after the compensatory growth (C) regime fish had fully compensated in growth such that their mean size was not significantly different from that of the *ad libitum* growth (A) regime fish.

The colour intensity of the males' red nuptial throat was measured using a protocol adapted from Frischknecht (1993) and successfully used in more recent studies such as Inness & Metcalfe (2008) and Lee et al. (2012). Each male was netted in turn from its nesting aquarium and temporarily placed on a white board which was used as a standard background. The left lateral surface of each male was then photographed alongside a colour chart. The digital photographs were taken using a tripod-mounted Panasonic LUMIX DMC-T225 digital camera which was kept at a fixed distance during all photographic sessions. The illumination was also kept constant across photographic sessions with two full spectrum daylight lamps placed at 45° at either side of the white board. The male throat colouration can fade after the males are taken out of water (Frischknecht 1993), although the strength of this effect is debatable (Laurin & Scott 2009). Nonetheless, to prevent their colouration fading, only one photograph of each male was taken at each time point. This reduced handling time to less than 60 seconds and reduced unnecessary stress to the fish. However, this method did not constrain the results, since previous work has found that a single photograph of the lateral surface of the males produces comparable results to photographing both the lateral and ventral sides (Braithwaite & Barber 2000).

Image analysis was performed using ImageJ 1.46 software (National Institutes of Health, USA). The red throat area of the male was cropped from each image according to the procedures described in Barber et al. (2000). Using the RGB Measure plug-in, the selected throat area obtained a score for redness, greenness, and blueness. To standardise for any confounding variation in light and tone between photographs, all throat values were divided by the colour level values obtained from the standard colour chart in each photograph. This procedure is fully described in Inness & Metcalfe (2008). The standardised intensity of the red throat of each male (R) was calculated as red_{STD}/ (red_{STD}+ $green_{STD}$+ $blue_{STD}$) where red_{STD}, $green_{STD}$ and $blue_{STD}$ represent the standardised values for the red, green and blue channels, e.g. red_{STD} = (mean intensity of red brightness of selected throat area)/(mean intensity of red brightness of selected red standard on colour chart). Therefore, a high R value would be obtained when a high proportion of the total image brightness was made up of the red channel (Barber et al. 2000).

4.3.4 Statistical analysis of nuptial colouration

The effect of both growth regime and dietary manipulation on the standardised red intensity of the male's nuptial throat colouration was analysed using a general linear mixed model (GLMM), with growth regime (A or C), carotenoid supplementation (H or L), resveratrol supplementation (R or N), and the time point from the commencement of the breeding season (Week 0, 5, 10 and 12) included as fixed effects. Fish length (manipulated length at the end of Period 1) was included as a covariate, plus all two-way interactions among variables. Fish identity was included as a random factor to control for repeated measures carried out on the same fish across the four different time points during the breeding season.

4.3.5 Statistical analyses of nest building

The effect of both growth regime and dietary manipulation on nest building rate, pre lag building rate and nest thread count were analysed separately using general linear models (GLM), with growth regime (A or C), carotenoid supplementation (H or L) and resveratrol supplementation (R or N) fitted as categorical explanatory variables. Manipulated fish length at the end of Period 1 and fish length just before the introduction of the nesting material (31st May 2011) were included as covariates, plus all two-way interactions among variables. The nest thread count was analysed as the total proportion of the one hundred threads provided which were used to complete the nest. These proportional scores were arcsine square root transformed prior to statistical analysis.

4.3.6 Statistical analyses of relationships between oxidative stress, nest building and throat intensity

The effects of nest building ability on the three elements associated with oxidative stress (GPx, SOD and protein carbonyl content) alongside growth regime and dietary manipulation was analysed using a general linear models (GLM), with growth regime (A or C), carotenoid supplementation (H or L) and resveratrol supplementation (R or N) fitted as categorical variables. GPx activity, SOD activity and protein carbonyl content were included as covariates, plus all two-way interactions among variables. In the general linear model, a single composite variable (PC1 - summarised in the 1st axis of the Principal Component Analysis of nest building parameters) was used as a measure of nest building

performance by combining the three nest building measures (nest building rate, pre-lag building rate and nest thread count) by running a principal components analysis (PCA) using a correlation matrix. The first principal component (PC1) explained 72.5% of the total variance in nest building ability. A positive value for PC1 indicates a male that utilised a larger number of threads to construct their nests and that completed their nests faster.

The effects of red throat intensity on the three elements associated with oxidative stress (GPx, SOD and protein carbonyl content) alongside growth regime and dietary manipulation was analysed using a general linear model (GLM), with growth regime (A or C), carotenoid supplementation (H or L) and resveratrol supplementation (R or N) fitted as categorical variables. GPx activity, SOD activity and protein carbonyl content were included as covariates, plus all two-way interactions among variables. To reduce the number of potential variables examined in the general linear model, a single score of red throat intensity was calculated by averaging the relative red throat intensity of the males across the final two stages of the breeding season when the males were in their full breeding condition. A male's relative throat intensity at each time point was expressed as a residual from the population mean.

All means are described with standard errors and all analyses in this present chapter were carried out using R (R Core Development Team, version 2.15.0). The function lme within the nlme package was used to fit the GLMM for nuptial colouration. The function prcomp was used to carry out the PCA using a correlation matrix. Significant results were defined as $p < 0.05$. Non-significant variables were sequentially dropped from each analysis so that the final models only included significant terms apart from main effects that occurred in significant two-way interactions.

4.4 RESULTS

4.4.1 Intensity of red nuptial throat colouration

The red throat intensity of the males was affected by whether they were fed a diet high or low in carotenoids, but this diet effect only became apparent in the later stages of the breeding season (Table 4.1; significant carotenoid × time interaction). Males fed a high carotenoid diet had significantly higher red throat intensity at week 10 (mean ± s.e. = 0.787

± 0.007) than males fed a low carotenoid diet (mean ± s.e. = 0.753 ± 0.009, t-test, t = 3.708, p <0.001). In addition, males fed a high carotenoid diet also had significantly higher red throat intensity at week 12 (mean ± s.e. = 0.840 ± 0.009) than males fed a low carotenoid diet (mean ± s.e. = 0.792 ± 0.009, t-test, t = 2.928, p = 0.005). However, there were no effects of carotenoid diet on red throat intensity at week 0 (high: mean ± s.e. = 0.691 ± 0.009, low: mean ± s.e. = 0.694 ± 0.009, t-test, t = -0.228, p = 0.82). Nor were there any effects of carotenoid diet in week 5 (high: mean ± s.e. = 0.683 ± 0.009, low: mean ± s.e. = 0.670 ± 0.008, t-test, t = 1.052, p = 0.30) (Figure 4.2).

There was also a significant effect of fish length at the end of the manipulation period on red throat intensity (GLMM, t_{107} = 5.9, p <0.0001) (Table 4.1). The males that were longer at the end of the manipulation period had redder throats than shorter males, regardless of the stage during the breeding season at which the red throat intensity was measured (Figure 4.3). However, there were no differences in red throat intensity in relation to growth regime or whether resveratrol was present or absent in the diet.

Table 4.1 Results of a general linear mixed model examining standardised red throat intensity in relation to growth regime, carotenoid supplementation, resveratrol supplementation and manipulated length after period 1. Fish identity was included as random factor.

Final model	Estimate	SE	DF	t	p
Carotenoid (L)	-0.003	0.011	107	-0.238	0.813
Time	0.012	0.001	209	13.045	<0.0001
Manipulated length	0.006	0.001	107	5.998	<0.0001
Regime (R)	0.006	0.008	105	0.759	0.450
Resveratrol (Y)	0.010	0.008	106	1.138	0.218
Carotenoid (L) × Time	-0.003	0.001	209	-2.638	0.009

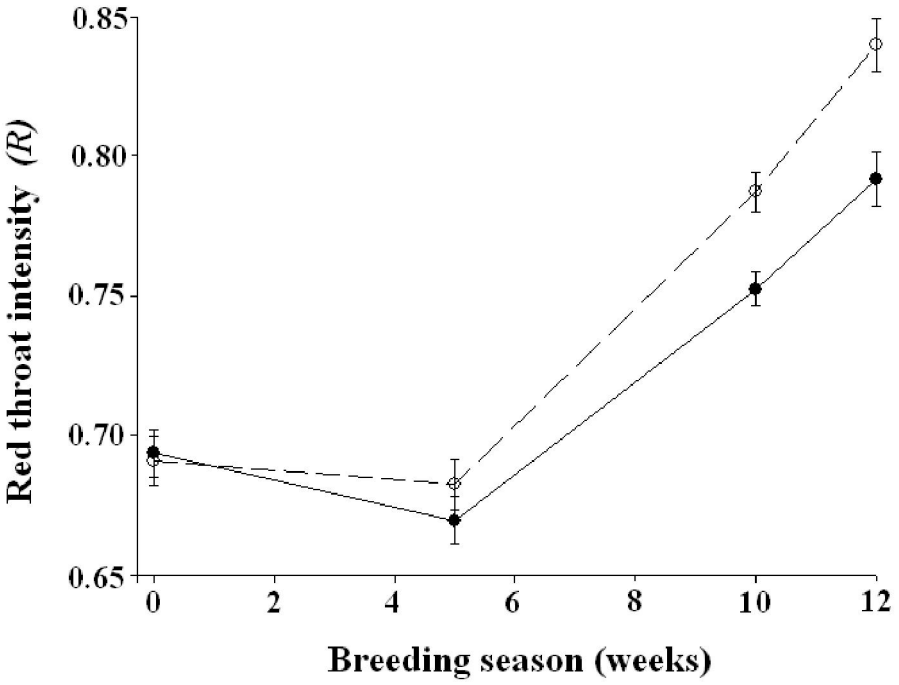


Figure 4.2 Mean ± s.e. standardised red throat intensity (*R*) of male three-spined sticklebacks in relation to level of carotenoids in their diet (high, open and dashed; low, closed and solid) and the stage of the breeding season. Growth regime and resveratrol supplementation groups were combined to illustrate the effect of carotenoid supplementation only.

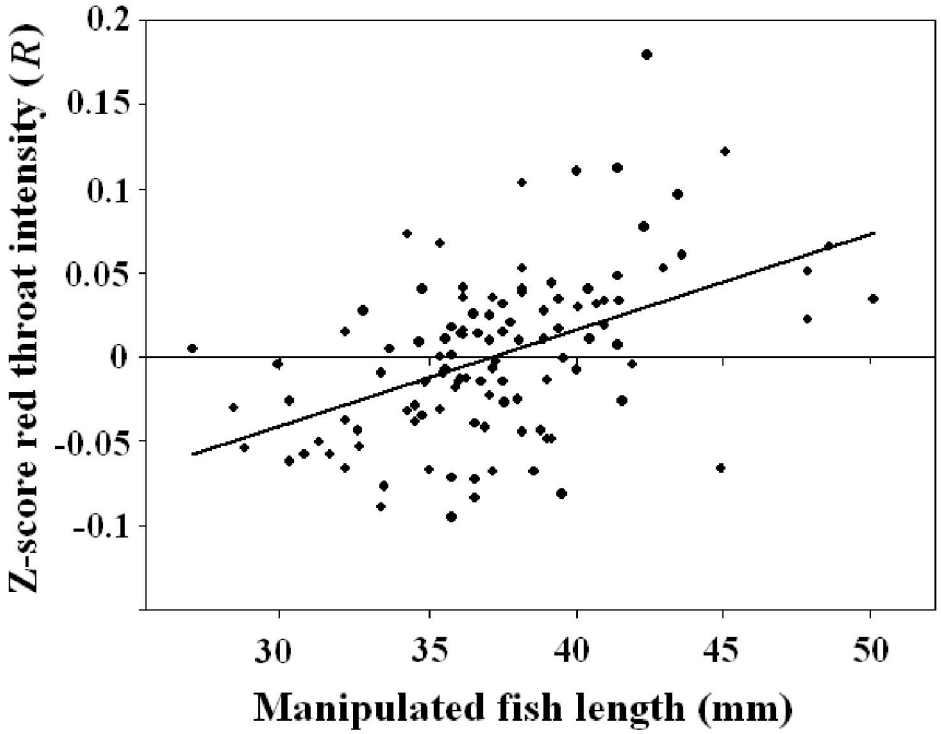


Figure 4.3 Relationship between fish length at the end of the manipulation period (Period 1) and mean z-score of standardised red throat intensity (*R*) across the four time points measured during the breeding season (adjusting for variation in mean redness over the season) for each individual and each of the four time points measured during the breeding season. The line (for illustration only) is fitted from a linear regression; analysis in table 4.1 takes account of repeated measures from each male.

4.4.2 Aspects of nest building

The males that were fed a low carotenoid diet took a significantly longer time to begin nest building than males fed a high carotenoid diet (GLM, $t_{75} = 2.61$, $p = 0.011$) (Figure 4.4). There was no difference in the time taken to begin nest building in relation to growth regime or whether resveratrol was present or absent in the diet. The low carotenoid males also took a significantly longer time to complete nest building than males fed a high carotenoid diet (GLM, $t_{75} = -2.87$, $p = 0.005$) (Figure 4.4). There was again no difference in the time taken to complete nest building in relation to growth regime or whether resveratrol was present or absent in the diet. There was no difference in the total number of threads used to construct the nest in relation to growth regime, whether resveratrol was present or absent in the diet, or whether carotenoids were fed in a high or low dose in the diet (Figure 4.5).

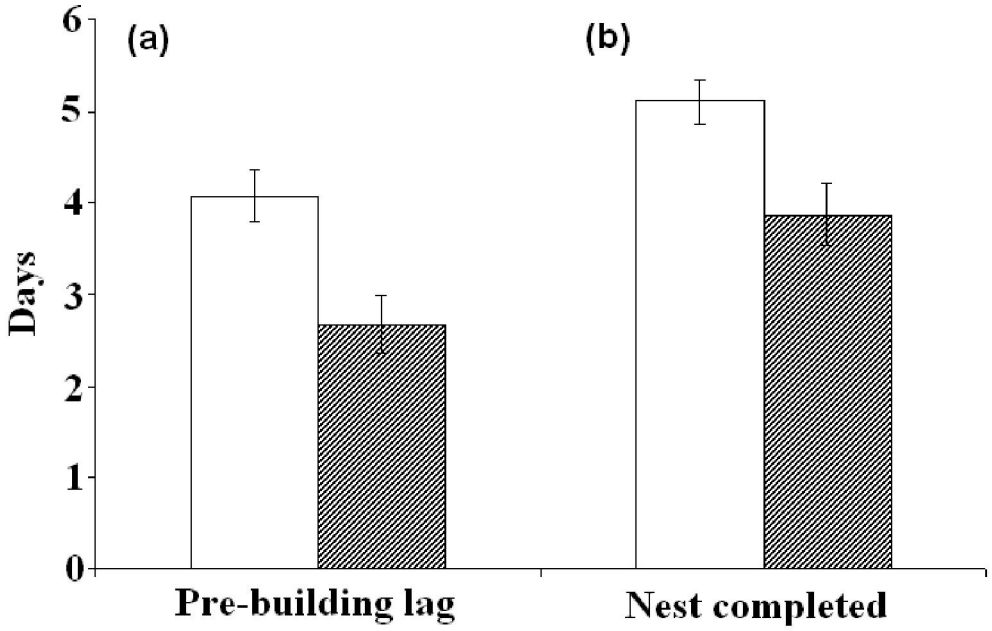


Figure 4.4 Mean ± s.e. total number of days to a) begin construction of the nest ("pre-building lag") and b) complete nest building (inclusive of the "pre building lag"), in relation to level of carotenoids in the diet (hashed bars: high carotenoid diet, open bars: low carotenoid diet); n = 76 males. Resveratrol supplementation and growth regime groups were combined to illustrate the effect of carotenoid supplementation only.

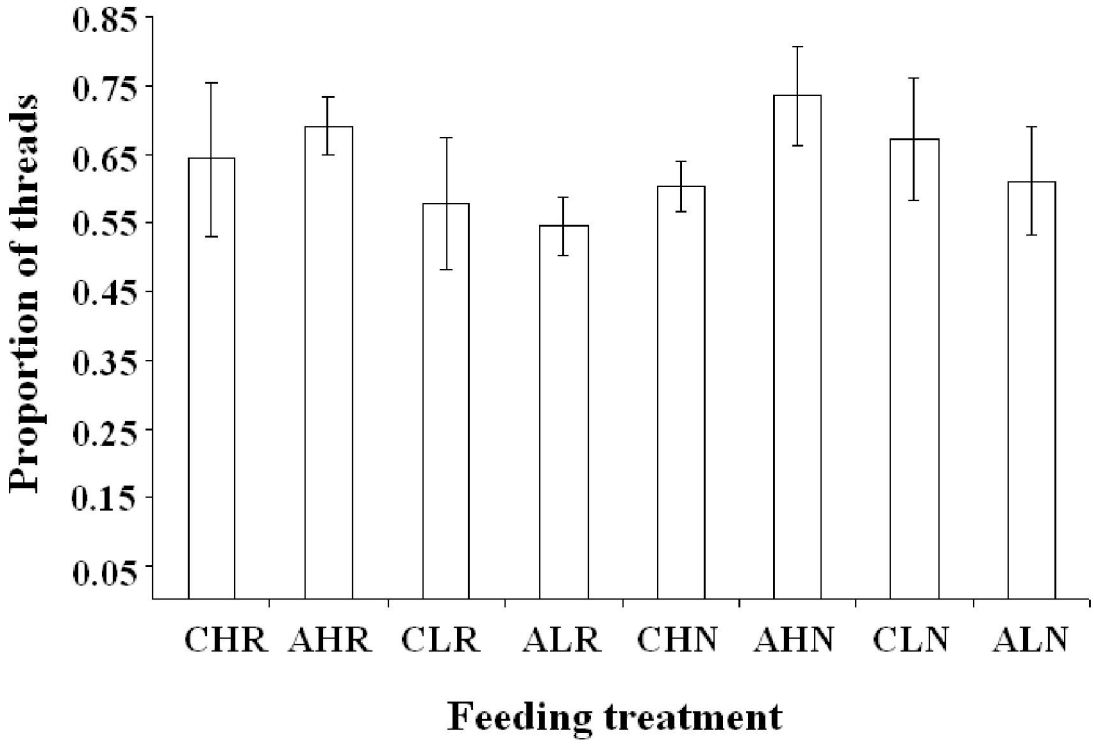


Figure 4.5 Mean ± s.e. proportion of threads used to complete nest building in relation to feeding treatment group. High carotenoid + resveratrol compensatory growth, high carotenoid + resveratrol *ad libitum* growth, low carotenoid + resveratrol compensatory growth, low carotenoid + resveratrol *ad libitum* growth, high carotenoid no resveratrol compensatory growth, high carotenoid no resveratrol *ad libitum* growth, low carotenoid no resveratrol compensatory growth and low carotenoid no resveratrol *ad libitum* growth denoted CHR, AHR, CLR, ALR, CHN, AHN, CLN and ALN respectively; n = 76 males.

4.4.3 Relationships between nest building performance, throat intensity and oxidative stress

Nest building performance (PC1) was negatively associated with glutathione peroxidase activity (GLMM, t_{61} = -2.936, p = 0.005; Table 4.2), but was unrelated to superoxide dismutase activity and protein carbonyl content (Table 4.2, Figure 4.6a and e). Males that built their nests faster thus had lower GPx activity at the end of the breeding season (Figure 4.6c). No relationship was found between average red throat intensity and the three oxidative stress measurements; GPx activity, SOD activity and protein carbonyl content (Table 4.3, Figure 4.6b, d and f). Males that had redder throats built their nests faster during the breeding season and also utilised a larger number of threads to construct their nests (GLM, t_{61} = 2.60, p = 0.012) (Figure 4.7).

Table 4.2 Results of a general linear model examining male nest building performance (PC1) in relation to growth regime, carotenoid supplementation, resveratrol supplementation, and three oxidative stress measurements; GPx activity, SOD activity and protein carbonyl content.

Final model	Estimate	SE	*t*	*p*
GPx	-1.085	0.370	-2.936	0.005
Protein Carbonyls	-0.162	0.195	-0.830	0.410
SOD	0.357	0.573	0.623	0.536
Carotenoid (L)	-0.859	0.380	-1.916	0.060
Resveratrol (Y)	0.269	0.365	0.736	0.465
Regime (R)	-0.054	0.405	-0.133	0.895

Table 4.3 Results of a general linear model examining residual red throat intensity (*R*) in relation to growth regime, carotenoid supplementation, resveratrol supplementation, and three oxidative stress measurements; GPx activity, SOD activity and protein carbonyl content.

Final model	Estimate	SE	*t*	*p*
GPx	-0.007	0.006	-1.160	0.25
Protein Carbonyls	-0.003	0.003	-0.891	0.38
SOD	0.009	0.009	1.054	0.30
Carotenoid (L)	-0.022	0.005	-3.948	0.0002
Resveratrol (Y)	0.005	0.006	0.879	0.38
Regime (R)	-0.001	0.006	-0.193	0.85

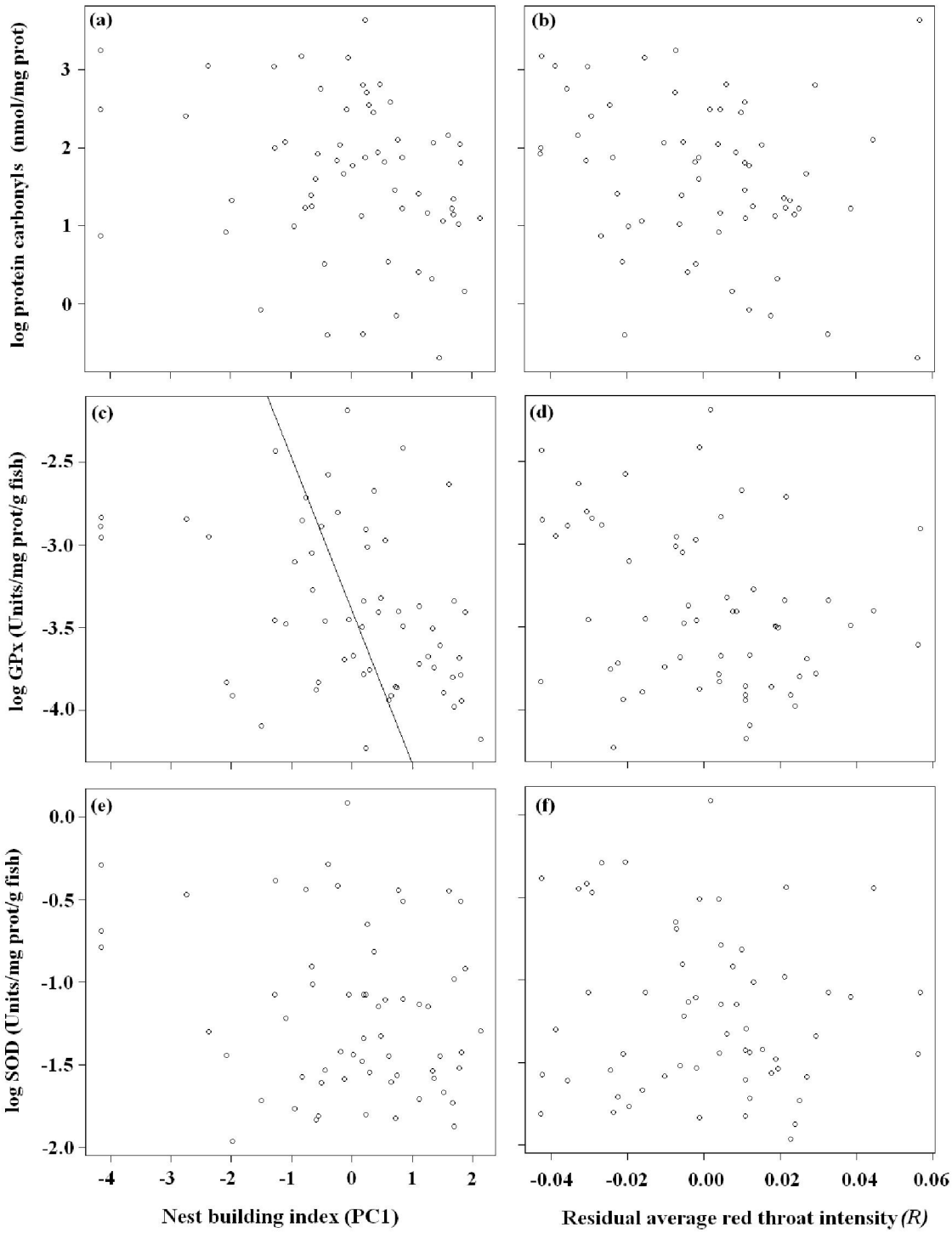


Figure 4.6 Relationships between reproductive parameters (nest building performance as measured by the 1st axis of a PCA analysis (PC1, where positive values indicate faster nest building and a male's use of a larger number of threads to construct their nests) and residual average red throat intensity) and measures of oxidative stress (protein carbonyl content, glutathione peroxidise (GPx) activity and superoxide dismutase (SOD) activity). Panel (c) illustrates a significant negative relationship between GPx activity and the nest building performance index (PC1). The linear regression shown here is for illustrative purposes only (see text for full statistical analysis). Panels (a), (b), (d), (e) and (f) were not statistically significant (see Table 4.2 and Table 4.3 for details); n= 61 males.

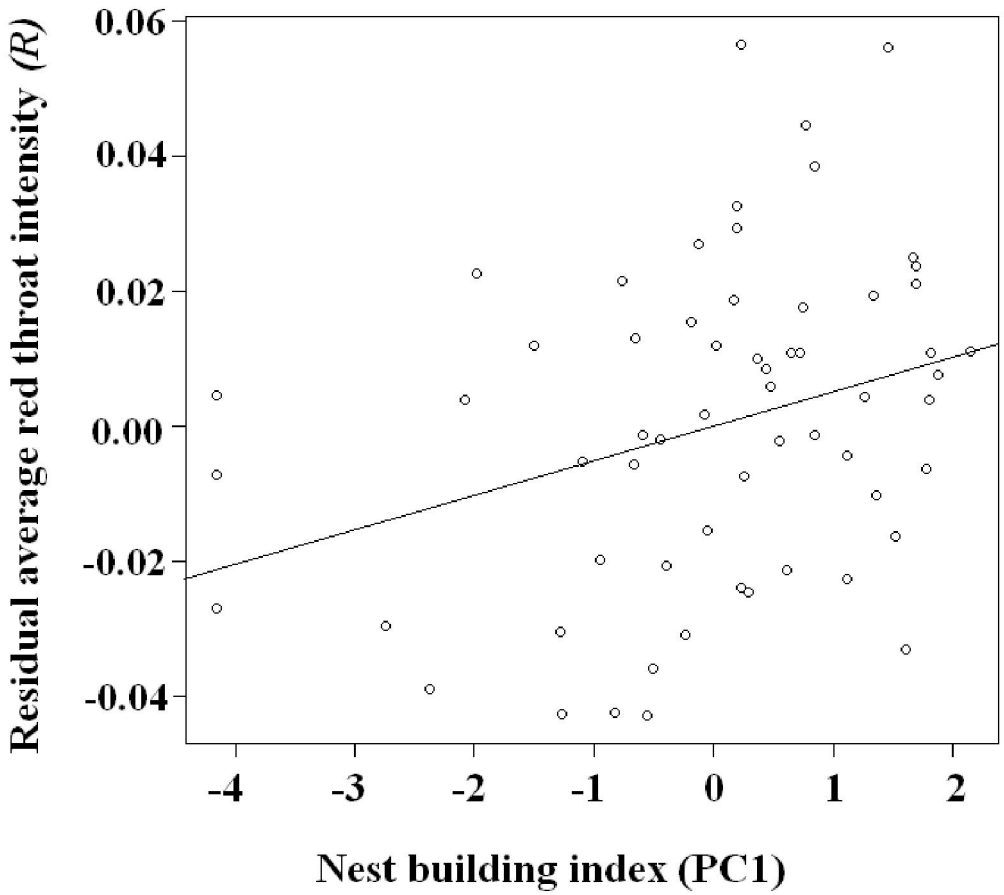


Figure 4.7 Relationship between nest building performance (PC1 where positive values indicate faster nest building and a male's use of a larger number of threads to construct their nests) and red throat intensity for each individual The linear regression shown here is for illustrative purposes only (see text for full statistical analysis); n = 61 males.

4.5 DISCUSSION

4.5.1 Red throat intensity and oxidative stress

To our knowledge, this is the first study to investigate the combined dietary effects of carotenoids (two non-vitamin lipophilic compounds) and resveratrol (a non-vitamin hydrophilic compound) on mediating the expression of a carotenoid-based sexual ornament after a period of compensatory growth. The degree of expression of the sexual ornament in the male three-spined sticklebacks varied with regard to the stage of the breeding season. At the onset of the breeding season (week 0 and week 5), there were no differences in the expression of the nuptial colouration between diet treatments. Perhaps this is to be expected, since in species such as the stickleback where male reproductive success is tightly linked to sexual signal investment, males can compensate for previously poor resource availability once conditions have improved by disproportionately investing in the

expression of the sexual ornamentation at the expense of other traits (Lindström et al. 2005). For instance, male three-spined sticklebacks provided with a limited supply of dietary carotenoids have been found to continue to invest a plentiful supply of carotenoids into their sexual signal at the expense of their body carotenoids (Pike et al. 2007a), despite these body carotenoids being presumed to play vital roles in other important physiological functions such as in the detoxification system (Olson & Owens 1998; Bertrand et al. 2006b; Pike et al. 2007a). Since reproduction imposes considerable energy expenditure for male three-spined sticklebacks (Huntingford et al. 2001), distributing a limited carotenoid resource in this manner may increase their susceptibility to oxidative stress if carotenoids do indeed play a role as an antioxidant (Cubillos & Guderley 2000). In alignment with this hypothesis, in the present study the male three-spined sticklebacks fed a low carotenoid diet did indeed have higher oxidative damage in terms of protein carbonyl content at the end of the breeding season. This suggests that a disproportionate investment in the expression of their sexual signal at the beginning of the breeding season may have come at a cost of greater oxidative damage towards the end of the season. A similar outcome was found in Pike et al. (2007a), where male three-spined sticklebacks also fed on a low carotenoid diet were found to be more susceptible to oxidative stress, as revealed by their elevated malondialdehyde levels (MDA) which were used as a measure of lipid peroxidation.

However, this dishonesty in sexual signal investment is less likely to occur with the progression of the breeding season as survival decreases (Lindström et al. 2009). Indeed, it was found that high carotenoid fed males had brighter throat colouration in comparison with low carotenoid fed males during two important stages of the breeding season (week 10 and 12). This corresponds to the period after nest building, which is a crucial stage of the breeding season since the intensity of the male's sexual ornament plays a primary role in attracting females to mate, which in turn results in the female depositing her full compliment of fertilised eggs in the male's nest (Pike et al. 2007a). Female three-spined sticklebacks most often prefer males with a brighter throat (Milinski & Bakker 1990). In the present study, perhaps the low carotenoid fed males were no longer able to maintain their dishonest sexual signal after the energetically expensive task of nest building. These results would also suggest that the high carotenoid fed males with the brighter throats were honestly signalling their enhanced ability to resist oxidative stress, since they had significantly less protein carbonyl content than the low carotenoid fed males and there was a significant correlation between red throat intensity and protein carbonyl content during

these two crucial stages of the breeding season (week 10 and week 12). The low carotenoid fed males not only had a significantly reduced intensity of throat coloration but also had higher protein carbonyl content by the end of the breeding season, possibly because their lower level of carotenoids resulted in their being less able to resist oxidative damage to proteins. This would suggest that carotenoids played an important part as a dietary antioxidant in this present study.

During the breeding season, upregulation of endogenous antioxidant defences can come as a result of demanding activities associated with reproduction, such as investment in sexual ornaments (Garratt & Brooks 2012). However, in the present study males with brighter throat colouration (high carotenoid fed males) had reduced GPx activity at the end of the breeding season which suggests that these males may have been in a restored and balanced redox state at the time of sampling. The males that had paler throat colouration were also the males that performed poorer in nest building. These males also had higher GPx activity suggesting that they may have strategically reduced their reproductive investment in order to preserve antioxidant capacity.

Resveratrol had no effect in influencing the intensity of the red throat signal at any of the four stages measured during the breeding season. These findings are inconsistent with the results of previous experimental studies that have found that provision of non-pigmentary antioxidants can enhance the expression of carotenoid-based sexual ornamentation in zebra finches, yellow-legged gulls *Larus michahellis* and also in three-spined sticklebacks (Bertrand et al. 2006b; Pike et al. 2007b; Pérez et al. 2008). The suggestion that carotenoid-based sexual signals advertise not the carotenoid themselves but other colourless antioxidants, does not hold with regard to the results of the present study which included treatments in which fish received a supplement of resveratrol as a potential *in vivo* antioxidant (Hartley & Kennedy 2004). The present results would suggest that resveratrol does not play a role in mediating the expression of the male's sexual ornament in sticklebacks. This may be because resveratrol does not have comparable antioxidant properties to the alternative dietary antioxidants used in these previous studies such as vitamin E (Pérez et al. 2008). It is also possible that the supplementation of resveratrol may have stimulated hormesis in the three-spined sticklebacks, as this compound is not found naturally in their diet or perhaps they do not assimilate resveratrol from their diet. Indeed, resveratrol has been reported to induce antioxidant defence indirectly through changes in gene expression in yeast cells and human adipocytes by hormetic ROS accumulation

(Escoté et al. 2012). Therefore, in the present study resveratrol supplementation may not have resulted in direct antioxidant protection but rather induced antioxidant protection indirectly by activating the transcription of detoxifying genes. This may explain the lack of effect of resveratrol in influencing throat colouration (which was linked to oxidative stress status) in the three-spined sticklebacks in the present study.

4.5.2 Nest building performance and oxidative stress

It has been proposed that male three-spined sticklebacks attract females not only by their red nuptial throat colouration but also by the quality of their nests, which thus act as an extended sexual ornament (Barber et al. 2001). This hypothesis is also supported in other male nest-building species including the fifteen-spined stickleback *Spinachia spinachia*, the stream goby *Rhinogobius* sp. and also the house wren *Troglodytes aedon* (Östlund-Nilsson 2001; Takahashi & Kohda 2002; Ólafsdóttir et al. 2006; Eckerle & Thompson 2006). It has been suggested that nest construction can provide females with important information of a male's quality, comparable to that provided by his sexual ornament (Barber et al. 2001; Rushbrook et al. 2008). Indeed, the results of this present study found that the males that built their nests faster also displayed brighter nuptial throat colouration. However, nuptial throat colouration was not correlated with the number of threads that these males incorporated into building their nests. The number of threads used to build their nests may in fact be a more important aspect of nest building that a female uses to evaluate a potential mate as she may not have observed how fast the males completed nest building. In hindsight, it would have been interesting to evaluate any differences in nest structure as this has been previously proposed to act as a reliable indicator of male quality in male three-spined sticklebacks (Rushbrook et al. 2008). It is noteworthy that the correlations found between measurements of throat colouration and oxidative stress were also mirrored in the correlations found between measurements of nest building performance and oxidative stress. These correlations suggest that males in good condition (in terms of reduced oxidative stress) were better able to undergo the physical activity required to build their nests. In support of this, it has recently been shown that male three-spined sticklebacks that had exhibited compensatory growth (which has been proposed to increase oxidative stress) took longer to complete nest building than continuous growth peers (Lee et al. 2012), although it should be noted that oxidative stress was not measured in that study (Lee et al. 2012).

4.5.3 Limitations and conclusions

The present study provides support for the recent consensus that the timing of assessing oxidative stress status in relation to investment in reproduction is important when interpreting results. The issue of whether oxidative stress is measured before or after the reproductive event has been highlighted recently as a plausible explanation for why oxidative stress and reproduction are often found to be either positively or negatively associated with one another (Stier et al. 2012). In the present study, it is important to note that there is a discrepancy between the timings of the measurements of nuptial throat colouration and nest building performance compared to those of oxidative stress: the fish were culled for their oxidative stress measurements 10 weeks after the completion of nest building and 7 weeks after the final measurement of nuptial throat colouration. Therefore, the timings of these measurements prevents ascertaining whether the level of oxidative stress incurred by the fish was higher as a consequence of compensatory growth prior to the breeding season which consequently constrained investment in sexual ornamentation and nest building. Nevertheless, the results do suggest that the levels of oxidative stress status came at a cost of the degree of investment in reproduction and this was mediated by the degree of carotenoid but not resveratrol supplementation.

The present study was carried out in a controlled laboratory environment and therefore the experimental animals did not bear the same potential costs of compensatory growth that would be expected in the wild such as avoiding risk of predation (Hector & Nakagawa 2012). The absence of factors such as these may explain the absence of the importance that growth regime had on influencing reproductive investment in the present study. For instance, one recent study in three-spined sticklebacks did find significant costs to reproductive investment following compensatory growth (Lee et al. 2012). However, it should be noted that strongest negative effects of compensatory growth on reproductive investment were achieved when photoperiod manipulations were used in order to reduce the perceived time available to complete this growth compensation before the onset of the breeding season (Lee et al. 2012). Perhaps such strong effects were not achieved in the present study due to the sticklebacks having a larger time period available in order to achieve and recover from full growth compensation prior to commencing the breeding season. Nevertheless, the findings of the present study do suggest that carotenoids but not resveratrol played an integral role in maintaining reproductive investment whilst combating the costs associated with a period of compensatory growth in early life.

CHAPTER 5 – A NON-CAROTENOID DIETARY SUPPLEMENT DOES NOT INFLUENCE MALE ATTRACTIVENESS IN A FEMALE MATE CHOICE PARADIGM BUT DOES INCREASE FEMALE CHOOSINESS

5.1 ABSTRACT

Many animals, including male sticklebacks exhibit a compensatory growth response to eliminate the reproductive costs of a smaller body size which are linked to reductions in sexual attractiveness to females. However, this investment in compensatory growth may come at a cost to other important sexually-selected traits which may reduce a male's sexual attractiveness. The potential costs associated with compensatory growth on female mate preference with regard to male sexual attractiveness have received little investigation to date. However, alongside body size, sexual ornamentation is known to influence female mate preference. In three-spined sticklebacks, males must obtain a plentiful supply of carotenoid in order to express their carotenoid-based nuptial colouration. However, one such additional factor which has been suggested to influence the intensity of carotenoid-based sexual signals is the availability of non-carotenoid dietary antioxidants. This chapter investigated the effects of a compensatory growth regime alongside a diet supplemented with resveratrol (a colourless antioxidant) on male sexual attractiveness by measuring female mate preference in a dichotomous choice design experiment. Additionally, the females were also subjected to the same diet treatments in order to assess whether this influenced female choosiness, as the process of mate choice can be energetically costly, and a female's active assessment and comparison among potential mates is likely to increase her susceptibility to oxidative stress. The mate choice trials found that female three-spined sticklebacks did not exhibit a mate preference for males that had received a supplement of resveratrol. Females were also unable to distinguish between males that had exhibited a compensatory growth regime in early life from those that had grown steadily. This suggests that the females did not receive any alternative (and potentially important) mate cues mediated by resveratrol that were independent of the carotenoid-based signal. However, females supplemented with resveratrol in the present study spent significantly more time associating with males than females that had not been fed resveratrol. This suggests that resveratrol may facilitate active choice in females through beneficial effects associated with cognition.

5.2 INTRODUCTION

5.2.1 Compensatory growth and sexual attractiveness

In many species, a male's adult body size is an important factor in determining his sexual attractiveness (Brown 1990; Reynolds & Gross 1992; Hasegawa et al. 2013). As a consequence, larger males are at an advantage during the breeding season when attempting to attract a mate (MacLaren et al. 2004; Basolo 2004; Labonne et al. 2009; Deb et al. 2012). In addition, a larger body size is also beneficial to male mating success during competitive interactions between males which is a common occurrence during breeding attempts (Andersson 1994; McLennan 2007; Sawadogo et al. 2013). Therefore, males that have incurred a poor start in life, in terms of reduced food availability, often exhibit compensatory growth once conditions have improved (Hassanabadi 2008; Inness & Metcalfe 2008). Compensated males can obtain a body size comparable to males with unrestricted growth in an attempt increase their sexual attractiveness to females (Birkhead et al. 1999; Blount et al. 2003a).

However, alongside body size there are multiple visual mating signals such as behaviour and sexual ornaments which are known to influence female mate preference (Candolin 2003). These alternative visual signals may be negatively affected by compensatory growth, as this is known to have detrimental effects on key traits such as reproductive investment, locomotor performance and rates of senescence (Inness & Metcalfe 2008; Lee et al. 2012; Lee et al. 2013). Therefore, exhibiting compensatory growth to eliminate the aforementioned costs of a smaller body size may come at a cost to other important sexually-selected traits which may reduce male sexual attractiveness. Despite this, the effects of compensatory growth on female mate preference with regard to male sexual attractiveness have remained relatively unexplored (Kahn et al. 2012). To date, the few studies that are available in the literature have produced conflicting results (Blount et al. 2003a; Walling et al. 2007; Kahn et al. 2012).

In female mate choice trials with male mosquitofish *Gambusia holbrooki*, females preferred to associate with males that had experienced continuous growth over males that had undergone compensatory growth earlier in life (Kahn et al. 2012). However, the exact visual cues the females were using to provide them with evidence of a male's developmental history were undetermined (Kahn et al. 2012). As early food restriction is

known to have negative effects on the relative length of a male's gonopodium, and as some species of poecillid fish display their gonopodia during courtship, it was suggested that this may have been used as a visual cue (Kahn et al. 2010; Kahn et al. 2012). It was also suggested that subtle differences in locomotion may have provided the females with a cue to a male's developmental history, since compensatory growth can negatively affect locomotion (Álvarez & Metcalfe 2005; Lee et al. 2010). Indeed, there is accumulating evidence to suggest that female mate preference is often based upon male locomotor behaviour (Byers et al. 2010). However, no measurements of differences in gonopodium length and locomotory behaviour between paired males were included in the study (Kahn et al. 2012).

In contrast to these findings, male compensatory growth had no effect on female mate preference in zebra finches *Taeniopygia guttata* (Blount et al. 2003a). Additionally, in female mate choice trials, compensatory growth did not lead to a reduction in male sexual attractiveness in green swordtails *Xiphophorus helleri* (Walling et al. 2007). However, at the time of testing not only had full compensation occurred in terms of body size but there were also no differences between control and compensated males in terms of sword length (Walling et al. 2007). Sword length is an important aspect of a male's phenotype which is used to attract females in this species (Basolo 1990; Rosenthal & Evans 1998), and it is likely that this cue prevailed during these mate choice trials resulting in the lack of female preference between control and compensated males. Since the reproductive success of many species is tightly linked to the development of sexual ornaments, it may be expected that female preference for control males over compensated males will only occur when compensatory growth has resulted in a reduced sexual ornament. As the expression of the sexual ornament was not reduced in compensated males in the previous studies by Blount et al. (2003a) and Walling et al. (2007), this may explain why female preference did not differ between control and compensated males.

Compensatory growth did not affect the expression of the carotenoid-based sexual signal in the male subjects used in the present study (see Section 4.4.1, Chapter 4). However, it is possible that the compensated males were only able to maintain the expression of their sexual ornament at the expense of their somatic health. Therefore, we can hypothesise that although compensated and control males may be superficially indistinguishable in terms of their sexual ornaments, females may use alternative cues that signal the quality of the males. For example, control males may be able to invest more in courtship displays in

comparison with compensated males. The present study hypothesises that females will invest more time associating with males that exhibited continuous and un-restricted growth in the *ad libitum* growth regime than with males that exhibited compensatory growth.

5.2.2 Colourless non-carotenoid antioxidants and sexual attractiveness

Carotenoid-based sexual signals are hypothesised to be strongly indicative of a male's quality to females as they influence female mate preference in numerous species, with females most often preferring males that display the most intense and elaborate carotenoid-based sexual ornaments (Kodric-Brown 1989; Milinski & Bakker 1990; McGraw & Blount 2009; Lindström et al. 2009). For example, in pairwise mate choice trials using three-spined sticklebacks, females preferred males that had been supplied with a greater access to carotenoids, indicating that females can make adaptive mate choice decisions based on a male's carotenoid status (Pike et al. 2007a). However, alongside a male's carotenoid status there is an array of additional factors which may indirectly influence the expression of the carotenoid-based sexual signal, or sexual attractiveness in general, and may therefore ultimately affect a female's mate choice decision.

One such potential factor which has been suggested to influence the intensity of carotenoid-based sexual signals is the availability of non-carotenoid dietary antioxidants (Bertrand et al. 2006b). This hypothesis was suggested after supplementation of the colourless dietary antioxidant melatonin was shown to enhance the red bill colouration in zebra finches (Bertrand et al. 2006b). However, it was acknowledged that alongside being a powerful antioxidant, melatonin also exhibits hormone properties which may have confounded its observed positive effects on the carotenoid-based colouration in zebra finches which were initially credited to its antioxidant properties (Anisimov 2003; Bertrand et al. 2006b).

Nevertheless, in support of this hypothesis, male three-spined sticklebacks provided with a high availability of dietary vitamins C and E produced brighter nuptial throat colourations in comparison with their male equivalents fed a diet low in vitamins C and E; these males were also preferred by females in size-matched mate choice trials (Pike et al. 2007b). However, the results of several subsequent studies in bird species have not supported the hypothesis (Karu et al. 2008; Orledge et al. 2012). These conflicting findings in the literature suggest that the supplementation of colourless antioxidants may have species-

specific effects on influencing carotenoid-based sexual ornaments. Alternatively, these discrepancies between findings may be due to differences in the particular antioxidants supplemented in the different studies or the types of carotenoid-based ornaments being tested (Karu et al. 2008).

In the present study, an alternative colourless antioxidant - resveratrol - was manipulated in the diet of three-spined sticklebacks in order to determine whether its availability mirrored the positive effects on female mate preference that were observed when dietary vitamins C and E were supplemented in three-spined sticklebacks (Pike et al. 2007b). However, in contrast to the findings in Pike et al. (2007b), where the supplementation of dietary vitamins C and E resulted in a more intense nuptial throat colouration, the supplementation of resveratrol did not positively influence the expression of the carotenoid-based signal in these three-spined sticklebacks (See Section 4.4,1 Chapter 4). Nonetheless, resveratrol supplementation may still influence female mate preference through alternative mate cues that are independent of the intensity of the carotenoid-based signal. As resveratrol is a proposed antioxidant (Gülçin 2010; Cai et al. 2011) it may affect other traits and behaviours which may be under assessment by females. For instance, in some three-spined stickleback populations, females show strong mate preferences for male courtship effort and body condition rather than for male nuptial colour intensity (Cubillos & Guderley 2000; Künzler & Bakker 2001; Boughman et al. 2005). The present study hypothesises that females will invest more time associating with males supplemented with resveratrol than males that have not received resveratrol.

5.2.3 Female choosiness

The process of mate choice can be energetically costly, and a female's active assessment and comparison among potential mates is likely to increase her susceptibility to oxidative stress (Byers et al. 2005; Vitousek et al. 2007; Toomey & McGraw 2012). Being gravid may also influence the energetic costs associated with mate choice. For example, gravid female three-spined sticklebacks can face reduced manoeuvrability and swimming ability (Milinski & Bakker 1992; Rodewald & Foster 1998). In Galápagos marine iguanas *Amblyrhynchus cristatus*, females that had experienced reduced food availability were in poorer condition and this resulted in less involvement in mate choice activity (Vitousek 2009). Therefore, in the present study, females that have experienced different development histories in terms of the quantity and quality of their diet may be expected to

show differences in mate choice behaviour. For instance, it has been suggested that carotenoid availability may directly alter physiological functions which consequently affect a female's investment in mate choice behaviour (Toomey & McGraw 2012). Indeed, captive female house finches *Carpodacus mexicanus* fed on a low carotenoid diet exhibited reduced mate choice behaviour in comparison with high carotenoid fed females (Toomey & McGraw 2012).

The present study hypothesises that females supplemented with dietary antioxidants - carotenoids and resveratrol - will invest more time in mate choice behaviour and will spend longer actively discriminating between the males. Conversely, low carotenoid-fed females that were not supplemented with resveratrol are hypothesised to be less active and less choosey.

5.2.4 Aims

The present study used a subsample of male subjects that had been reared on a diet low in carotenoids from early life that were part of a larger scale experiment which is described in Chapters 3 and 4. These low carotenoid-fed males had been subjected to either a compensatory growth regime or an *ad libitum* growth regime, and had either been supplemented with resveratrol or not from early life. The present study used data obtained from mate choice trials that tested the effects of compensatory growth and resveratrol supplementation on male sexual attractiveness. The mate choice trials used a subsample of female subjects that were also sourced from the same larger scale experiment described in Chapters 3 and 4. These females were drawn equally and randomly from the 8 feeding treatment groups which are described in Table 2.1, Chapter 2. The experimental design was such that it was also possible to determine whether a female's nutritional history (measured in terms of her feeding treatment group) influenced the time she spent actively choosing between males. The present study also investigated whether the male oxidative stress status influenced the female's mate preference. The oxidative stress measurements were carried out at the end of the mate choice trials and are described in Chapter 3.

5.3 METHODS

5.3.1 Source of fish for the female mate choice test

The fish used in the present study were male and female three-spined sticklebacks from the experiments described in Chapters 3 and 4. Data on the growth, reproductive investment and oxidative stress status of these fish are described in Chapters 3 and 4 (Figure 5.1). The mate choice trials occurred during the breeding season from 11-29 July 2011. The mate choice trials were performed in the same room as the tanks in which they had been previously held in order to reduce any confounding effects of changes in lighting and temperature on behaviour. As described in Chapter 2, the temperature and the photoperiod were adjusted to match the mean ambient conditions at the source river for that time of year. The photoperiod was achieved using fluorescent lighting and was controlled by electronic timers.

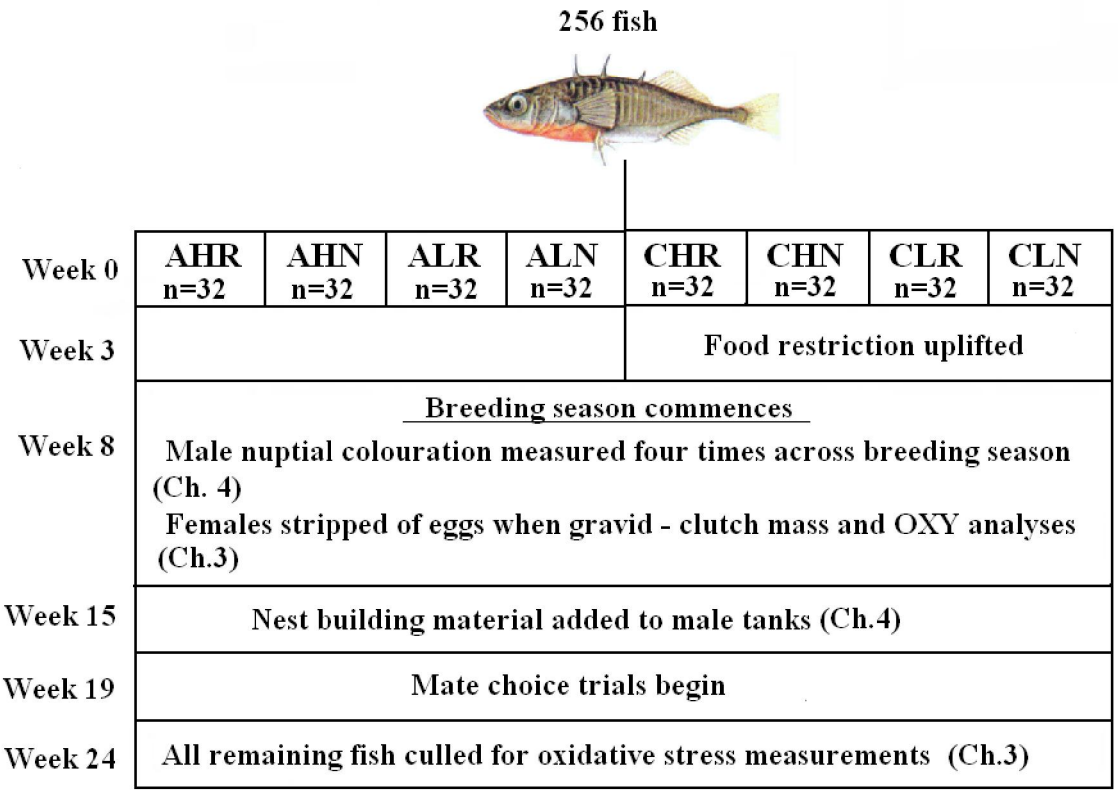


Figure 5.1 Schematic of experimental design. Fish were equally divided into the eight feeding treatments described in (Table 2.1, Chapter 2). At week 3 the food restriction was uplifted in the four compensatory growth regime fish (CHR, CHN, CLR and CLN) (See Chapter 3, Figure 3.2). At week 19, a subsample of males and females from Chapter 3 commenced mate choice trials. At week 24, all remaining fish were culled for oxidative stress measurements (See Chapter 3).

5.3.2 The female mate choice test

Female mate preference was tested using a standard dichotomous choice paradigm which is based on the assumption that the amount of time a female spends with either of two males corresponds to the strength of her preference for that particular male (Walling et al. 2007; Ward & McLennan 2009; Williams & Menderson 2011). The fully gravid female simultaneously assessed the males in pairs, with each male held in his own separate tank. Each gravid female was carefully placed into the middle of a plastic transparent experimental tank (33 x 18 x 19 cm) using a net and left to acclimatise for five minutes. This experimental tank was situated directly in front of the pair of tanks containing the two males. The position of the male tanks (in reference to the female experimental tank) was alternated between trials to reduce female compartment bias. The female's tank was divided into three zones: two equally sized "preference zones" (Z1 and Z2) at the front of the tank (closest to the male tanks) and a remaining zone at the back of tank which was classified as the "no choice" zone (see Figure 5.2). During acclimatization an opaque partition was slotted between the tanks so the females had no visual contact with the males prior to testing (males were unable to see each other at any time of the trial). To begin each trial, the partition was removed and the female's movements were filmed directly from above using a tripod-mounted mini DV camera recorder (PANASONIC-NV-GS60) at a fixed distance of 110cm. The trial lasted for five minutes, a time based on the duration used in other studies on this species (Milinski & Bakker 1990; Milinski et al. 2010). The total amount of time spent in each of the three zones was then recorded from the mini DV video tape recordings and used to calculate female mate preference. Female mate preference was expressed as the proportion of time the female spent in each preference zone (Z1 and Z2) within each five minute trial. Time spent in a zone was defined as the period from when the female's whole head entered to when it left the zone (Walling et al. 2007). Additionally, the proportion of time spent in both preference zones (Z1 and Z2) as opposed to time spent in the "no choice" zone was used as a measure of "choosiness" for each of the female sticklebacks during each trial; a low value would indicate that the female spent little time inspecting males. In this experiment, females were not allowed to spawn with their preferred male. However, previous studies in three-spined sticklebacks, green swordtails and guppies *Poecilia reticulata* have shown that the preferences displayed in dichotomous mate choice designs such as the present one are consistent with a female's mating preference when she is allowed to spawn (Kodric-Brown 1993; Cubillos & Guderley 2000; Kodric-Brown & Nicoletto 2001; Walling et al. 2010).

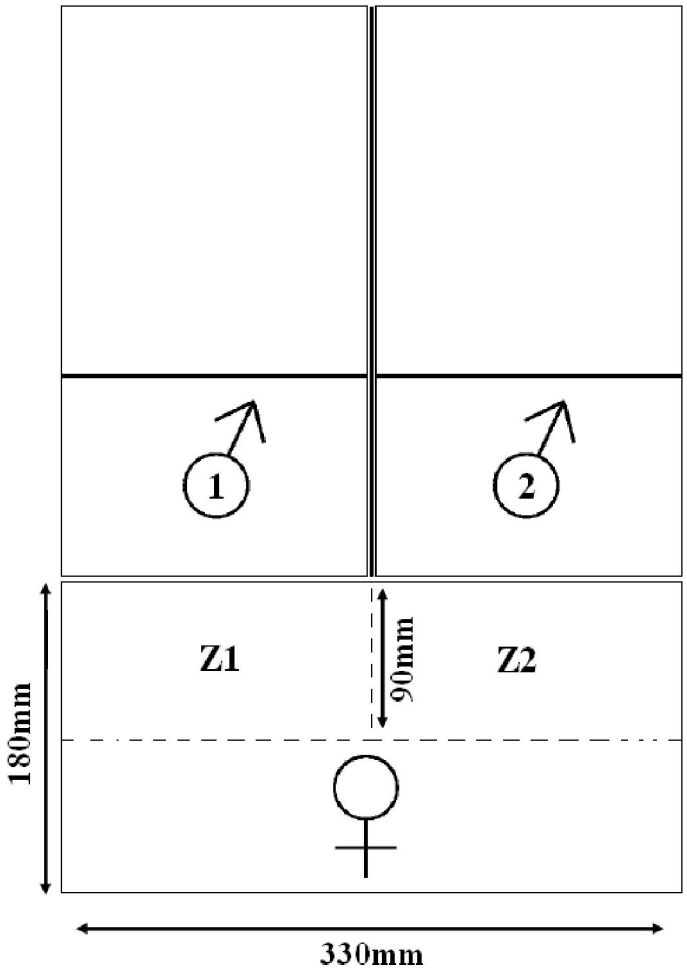


Figure 5.2 Female dichotomous mate choice aquarium set-up. Water depth was 160mm throughout all trials. A solid partition was slotted between the two long ends of the male tanks to prevent male-male visual contact. All three tanks were the same size in external dimensions, but partitions inserted into each male tank, 120mm away from each contact window, confined males so that the female was able to inspect them at a close distance. Preference for each male (1 or 2) was scored as the amount of time in each trial that a female spent in each associated preference zone (Z1 or Z2, respectively; the dashed lines represent the preference boundaries which were marked on the underside of the female's tank, while the area outwith these preference boundaries was regarded as the no-choice zone, 90mm away from the contact window).

5.3.3 The mate choice subjects

Female mate choice behaviour was quantified in 32 gravid females sampled equally from the 8 feeding treatment groups described in Table 2.1; Chapter 2. Gravid females were identified by the extension of their stomach region and from the opening of their cloaca. Reproductive status was further confirmed after the trials by stripping all 32 gravid females of their eggs. Their egg clutch mass was used as a measure of reproductive investment, as described in Chapter 3. The experiment used 17 pairs of sexually mature males, with no

male being used in more than one pair. Each of the 32 females was exposed to all 17 pair combinations in a randomised order, with each female exposed to two pairs of males each day (see Table 5.1 and 5.2 for pair combinations). All stimulus males had been on the low carotenoid diet, but were paired so that on each occasion the female either had a choice between a male on an *ad libitum* (A) or a compensatory growth (C) regime, or between a male that had received resveratrol (R) or not (N) in its diet. However, males within a pair were size-matched at the time of testing for mate choice, as there were no significant differences in terms of both standard length and mass between *ad libitum* and compensatory growth males by this point (see Figure 3.2; Chapter 3). These size-matched paired males were also closely colour-matched as neither resveratrol or growth regime influenced the expression of the carotenoid-based nuptial colouration in these three-spined sticklebacks (See Section 4.4.1, Chapter 4). The four low carotenoid treatment groups (ALR, ALN, CLR and CLN) which were examined in different pair combinations in these mate choice trials are fully described in Table 2.1; Chapter 2.

5.3.4 Measuring whether resveratrol influences female mate choice

A total of nine pairs of males were selected in which the only treatment difference between the two males was whether or not they had received resveratrol in the diet. Each of the 32 females was tested with each of these 9 pairs of males, resulting in a total of 288 trials. However, these were subdivided into the trials involving males on the *ad libitum* feeding regime (n = 4 pairs) and those on the compensatory growth regime (n = 5 pairs). This resulted in two separate analyses. The first analysis (A1) investigated female mate choice between the CLR and CLN treatment groups. The second analysis (A2) investigated female mate choice between the ALR and ALN treatment groups (see Table 5.1).

5.3.5 Measuring whether growth regime influences female mate choice

A further 8 pairs of males were selected that differed only in terms of their growth regime (*ad libitum* or compensatory growth); all 32 gravid females were again tested with each of these 8 paired males, yielding a further 256 trials. Analysis 3a (ALR versus CLR) and 3b (ALN versus CLN) were pooled together to statistically investigate female mate choice between the two growth regimes without taking into account whether or not resveratrol had been present in the diet, since it was found not to influence female mate choice in Analysis 1 and 2 (see Table 5.2).

Table 5.1 A tabulated summary of the mate choice design used to determine whether resveratrol influenced female mate choice. All 32 females had been exposed to the nine paired males by the end of the experiment. Each female participated in two mate choice trials (one from analysis A1 and one from A2) per day separated by at least 3 hours between the two trials.

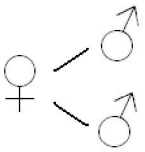
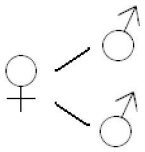
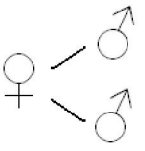
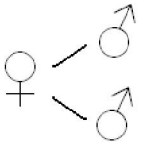
Analysis	Male group	Carotenoid	Resveratrol	Growth regime	Pairs tested
A1 	CLR	Low	Yes	Compensatory	5
	CLN	Low	No	Compensatory	
A2 	ALR	Low	Yes	*Ad libitum*	4
	ALN	Low	No	*Ad libitum*	

Table 5.2 A tabulated summary of the mate choice design used to determine whether male growth regime influenced female mate choice. All 32 females had been exposed to the eight paired males by the end of the experiment. Each female participated in two mate choice trials (one from each part of the analysis) per day separated by at least 3 hours between the two trials.

Analysis	Male group	Carotenoid	Resveratrol	Growth regime	Pairs tested
A3a 	CLR	Low	Yes	Compensatory	8
	ALR	Low	Yes	*Ad libitum*	
A3b 	CLN	Low	No	Compensatory	
	ALN	Low	No	*Ad libitum*	

5.3.6 Data organisation before statistical analyses

Females were only included in data analysis if they assessed both males by visiting both choice zones during the mate choice trial. Across the mate choice trials investigating the influence of resveratrol supplementation on female mate choice, three females were discarded from the analysis A1, and 7 females were discarded from the analysis A2 as they were completely inactive during the trial or spent 100% of the trial within the "no choice" zone. Additionally, across the mate choice trials investigating the influence of male growth regime on female mate choice, four females were discarded from the analysis A3 for the same reasons as above. This protocol resulted in a total of 310 trials being used for data

analysis investigating the effects of resveratrol supplementation and growth regime on female mate choice. However, inactive trials were included in the data analysis investigating whether a female's feeding treatment group influenced her "choosiness". In all non-excluded trials the time spent with each male was expressed as the proportion of time the female spent in his associated zone as a function of the total time spent with both males within each 5 minute trial. Therefore, the proportion of time spent in the "no choice" zone was not incorporated into the assessment of difference in time spent with the two males. These proportional scores were arcsine square root transformed prior to statistical analysis.

5.3.7 Statistical analysis of male resveratrol supplementation

For the A1 and A2 mate choice analyses, the effect of male resveratrol supplementation on the proportion of time the females spent with each male was analysed separately using two general linear mixed models (GLMM), with resveratrol supplementation (Y or N) included as a fixed effect. Female identity was included as a random factor. Male pair identity was also included as a random factor which was nested within female identity.

5.3.8 Statistical analysis of male growth regime

For the A3 mate choice analysis, the effect of male growth regime on the time the females spent with each male was analysed using a general linear mixed model (GLMM), with growth regime (A or C) included as a fixed effect. Female identification was included as a random factor. Male pair identification was also included as a random factor which was nested within female identification.

5.3.9 Statistical analysis of female "choosiness"

To determine whether a female's feeding treatment group influenced her "choosiness", a generalised linear mixed model was fitted with the Laplace approximation (GLZMM), with growth regime (A or C), resveratrol supplementation (R or N) and carotenoid supplementation (H or L) included as fixed effects and female identification as a random factor, plus all two-way interactions among variables. Female "choosiness" was in the form of proportional data and therefore measured with a binomial error. The proportion of

time spent choosing and the proportion of time spent not choosing were bound together into a two-vector response variable (Crawley 2007).

5.3.10 Statistical analysis of oxidative stress

To determine whether a male's oxidative stress status (measured in terms of glutathione peroxidase activity (GPx), superoxide dismutase activity (SOD) and protein carbonyl content) influenced the strength of the female's preference for that given male, a general linear mixed model was fitted (GLMM), with GPx, SOD, protein carbonyl content, and the chronological order that the analyses were undertaken were included as covariates, plus all two-way interactions among variables. Female identification was included as a random factor. Male pair identification was also included as a random factor which was nested within female identification. The methods used to measure oxidative stress are described in Chapters 3 and 4, respectively.

All means are described with standard errors and all analyses in this present chapter were carried out using R (R Core Development Team, version 2.15.0). The function lme within the nlme package was used to fit the GLMMs in Analyses 1 to 3 and the analysis investigating oxidative stress and red nuptial colouration. The function lmer within the lme4 package was used to fit the GLZMM investigating female "choosiness". Significant results were defined as $p < 0.05$. Non-significant variables were sequentially dropped from each analysis so that the final models only included significant terms apart from main effects that occurred in significant two-way interactions.

5.4 RESULTS

Across all mate choice trials in the four separate analyses, neither body weight (paired *t*-test: $t_{15} = 0.0049$, $p = 1.00$) nor length (paired $t_{15} = 0.66$, $p = 0.52$) differed between the two males on the different treatments.

5.4.1 Does resveratrol supplementation in males influence female mate choice?

Females did not differ in the mean percentage of time spent associating with low carotenoid males on a compensatory growth regime that were fed resveratrol (mean ± s.e. = 51.21 ± 2.72) versus not fed resveratrol (mean ± s.e. = 48.79 ± 2.72) e.g. CLR versus

CLN (GLZMM, $t_{103} = 0.58$, $p = 0.56$) (Figure 5.3; A1). Neither did females differ in the mean percentage of time spent associating with low carotenoid fed males on an *ad libitum* growth regime that were fed resveratrol (mean ± s.e. = 48.44 ± 4.36) versus not fed resveratrol (mean ± s.e. = 51.56 ± 4.36) e.g. ALR versus ALN (GLZMM, $t_{58} = 0.66$, $p = 0.51$) (Figure 5.3; A2).

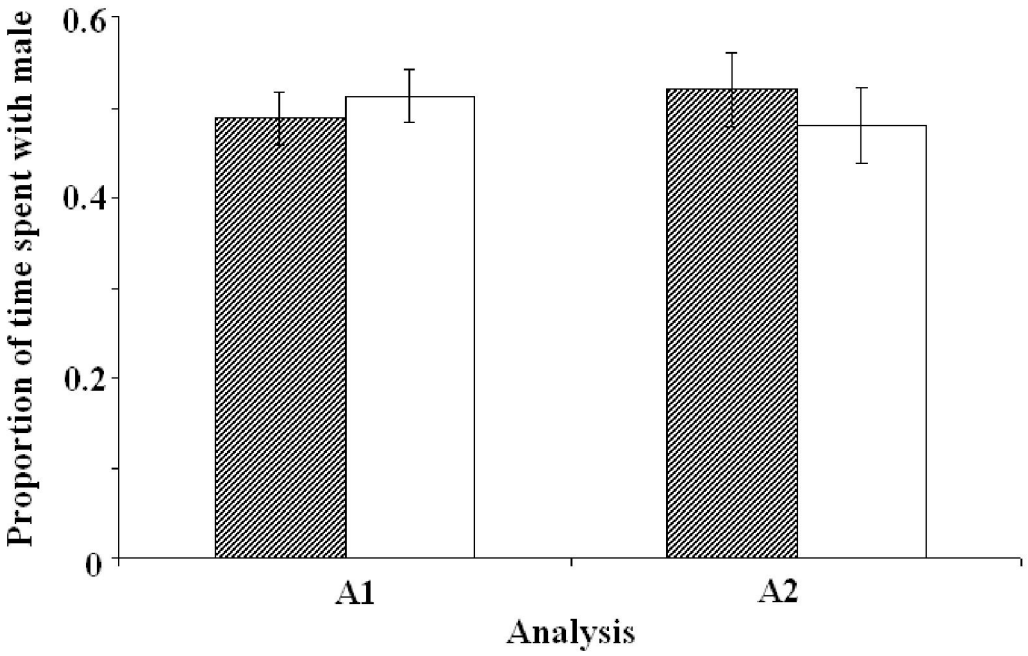


Figure 5.3 Mean ± s.e. proportion of time associating with male in his associated choice zone by all participating females (excluding omitted females) in analysis 1 (A1) and analysis 2 (A2), in relation to whether resveratrol was present or absent in the male diet (hashed bars: resveratrol , open bars: no resveratrol). There were no significant differences.

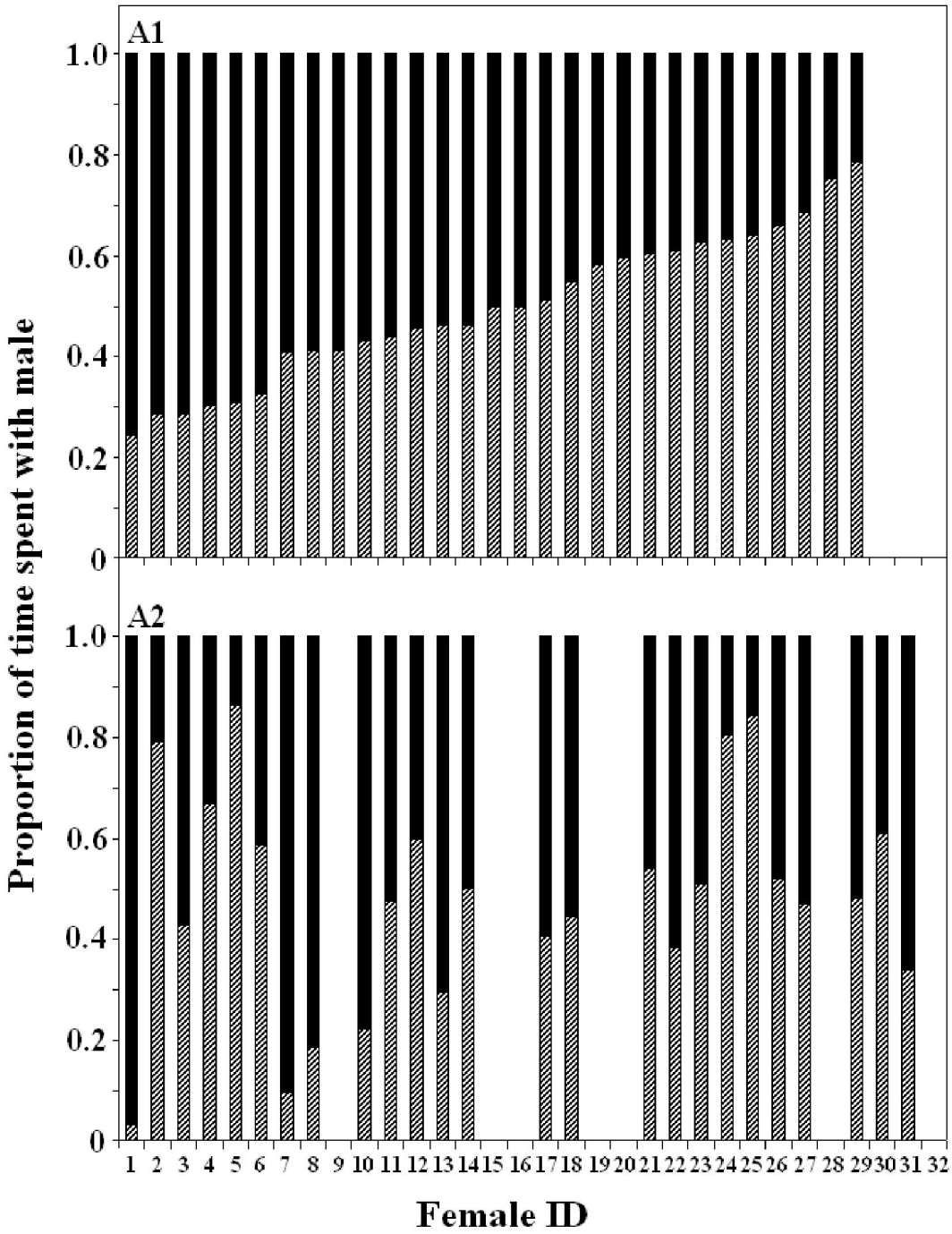


Figure 5.4 Graph A1 and A2 illustrating the lack of a consistent preference both between females for the males fed or not fed resveratrol in mate choice analysis A1 and A2, respectively. Dark portions of bars represent total time spent with males fed resveratrol while hashed portions of bars represent total time spent with males not fed resveratrol. For illustrative purposes, females are arranged in descending order of preference for male fed resveratrol (defined as proportion of time spent with male fed resveratrol minus proportion of time spent with male not fed resveratrol) in analysis A1, so that within-female comparisons can be made between all three analyses (A1-A3, this Figure and Figure 5.5). Missing bars indicate trials where the female did not visit both males.

5.4.2 Does male growth regime influence female mate choice?

Females did not differ in the amount of time spent associating with low carotenoid males that were fed under a compensatory growth (mean ± s.e. = 51.42 ± 2.52) versus *ad libitum* growth regime (mean ± s.e. = 48.57 ± 2.52) e.g. CLR and CLN versus ALR and ALN (GLZMM, t_{184} = -0.80, p = 0.43) (Figure 5.6).

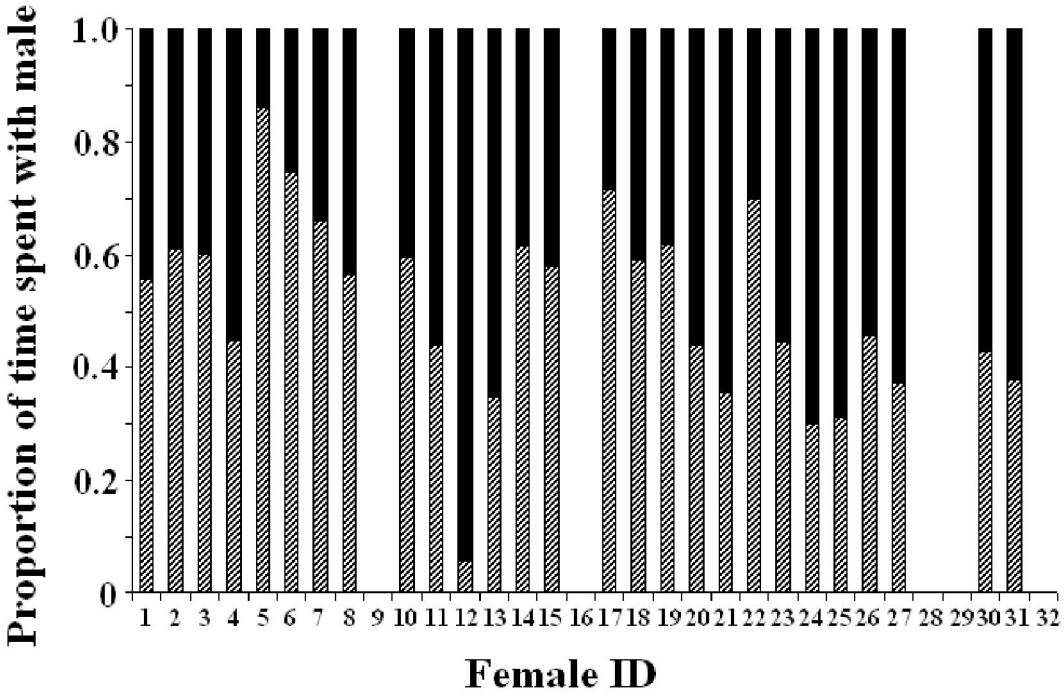


Figure 5.5 Graph illustrating the lack of a consistent preference between females for the males fed under an *ad libitum* or compensatory growth regime in mate choice analysis 3. Dark portions of bars represent total time spent with *ad libitum* males while hashed portions of bars represent total time spent with compensatory growth males. For illustrative purposes, females are arranged in the same order as in Fig. 5.4 A1 so within-female comparisons can be made between all three analyses (A1-A3, Figure 5.4 and this figure). Missing bars indicate trials where the female did not visit both males.

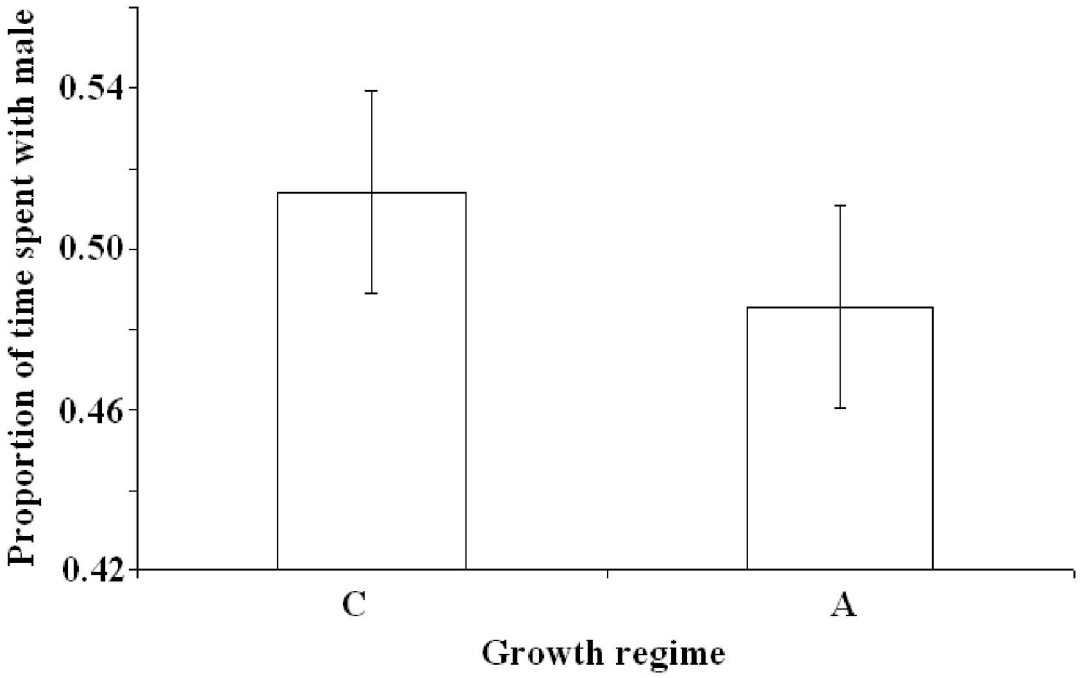


Figure 5.6 Mean ± s.e. proportion of time spent associating with each of two males by all participating females (excluding omitted females) in analysis 3a and 3b (combined to create A3) in relation to whether the males were fed under a compensatory growth (C) or an *ad libitum* (A) feeding regime. There were no significant differences.

5.4.3 Does a female's developmental and feeding history influence her "choosiness"?

A significant difference in female "choosiness" was found between the females fed resveratrol (R) and the females not fed resveratrol (N) (GLZMM, $z = -5.325$, $p < 0.001$), with females fed resveratrol (R) spending significantly more time choosing between males in terms of the proportion of time spent within the choice zones during a trial (mean ± s.e. = 0.56 ± 0.03) as opposed to proportion of time spent in the "no choice" zone (mean ± s.e. = 0.59 ± 0.03) (Table 5.3).

A significant interaction was found between growth regime and whether females were fed a diet low or high in carotenoids (GLZMM, $z = -3.629$, $p < 0.001$). If their diet was high in carotenoids, there was no difference in the females' "choosiness" regardless of whether they had an *ad libitum* growth (mean ± s.e. = 0.552 ± 0.04) or a compensatory growth regime (mean ± s.e. = 0.524 ± 0.04, t-test, $t = 0.489$, $p = 0.625$) (Figure 5.7). There were also no differences in the females' "choosiness" within the low carotenoid group between the compensatory growth regime (mean ± s.e. = 0.470 ± 0.04) and *ad libitum* growth regime (mean ± s.e. = 0.659 ± 0.04, t-test, $t = 1.511$, $p = 0.132$) (Figure 5.7). Regardless,

there is a stronger difference in "choosiness" between compensatory growth and *ad libitum* growth regime females in the low carotenoid group.

Table 5.3 Results of a generalised linear mixed model examining female choosiness in relation to growth regime, carotenoid supplementation and resveratrol supplementation.

Final model	Estimate	SE	*z*	*p*
Growth regime (C)	-0.235	0.963	-0.244	0.807
Carotenoid (L)	-2.187	0.898	-2.436	0.015
Resveratrol (Y)	-3.187	0.599	-5.323	<0.001
Carotenoid(L) × Regime(C)	4.206	1.159	3.629	<0.001

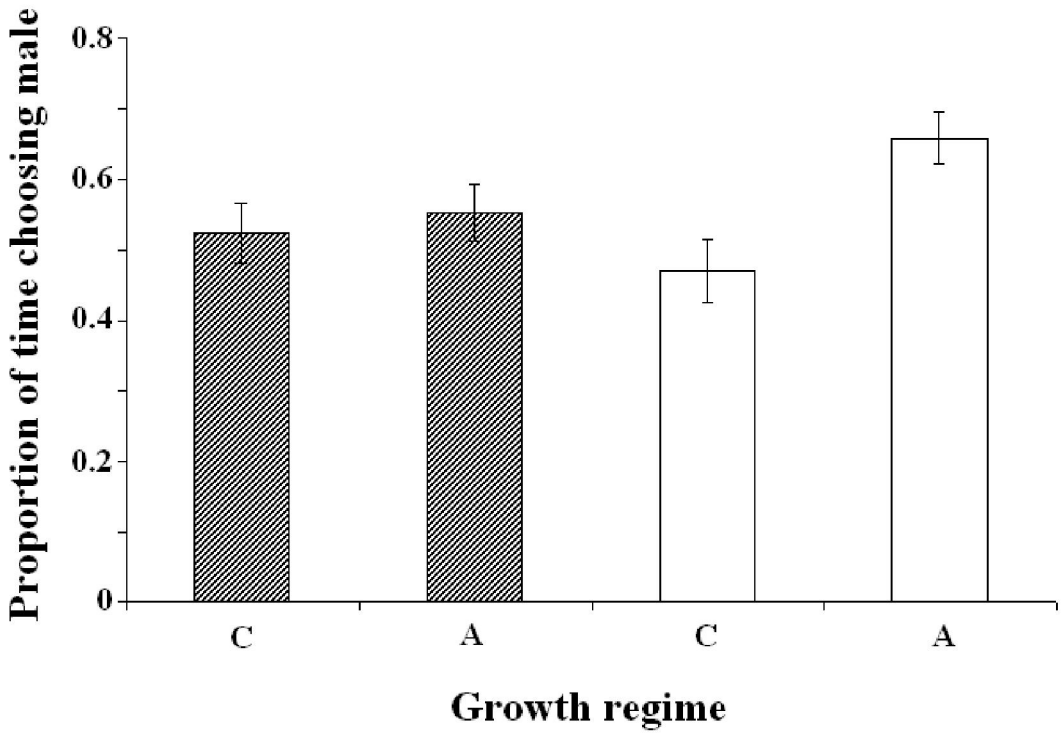


Figure 5.7 Mean ± s.e. proportion of time spent choosing between males in relation to growth regime (hashed bars: high carotenoid diet, open bars: low carotenoid diet). Compensatory growth regime and *ad libitum* growth regime are denoted C and A, respectively.

5.4.4 Does male oxidative stress status influence the strength of the female's mate preference in these low carotenoid males?

Male GPx activity was found to be associated with the strength of a female's preference (GLMM, t_{479} = 2.495, p = 0.013), with females preferring males with a higher GPx activity. However, the strength of female preference for each male was not influenced by SOD activity (Table 5.4). A significant interaction was found between male protein carbonyl content and the chronological order of the analyses that the females were undertaking (GLMM, t_{479} = -2.370, p = 0.018), with a reduction of the importance of male protein carbonyl content influencing female mate preference the further into the mate choice analyses the females were (Table 5.4).

Table 5.4 Results of a general linear mixed model examining female preference in relation to male oxidative stress status in terms of GPx activity, SOD activity and protein carbonyl content.

Final model	Estimate	SE	DF	*t*	*p*
GPx	1.359	0.545	479	2.495	0.013
Carbonyls	0.012	0.006	479	1.987	0.048
Analysis number	0.004	0.021	479	1.954	0.051
SOD	-0.047	0.141	478	-0.336	0.737
Carbonyls × Analysis number	-0.005	0.002	479	-2.370	0.018

5.5 DISCUSSION

5.5.1 Did a male's intake of resveratrol influence female mate choice?

The mate choice trials from the present study have demonstrated that female three-spined sticklebacks did not exhibit a mate preference for males that had received a supplement of resveratrol. This finding is in contrast to the results of a similar study, also in three-spined sticklebacks, which demonstrated that females did show a mate preference for males supplemented with two other non-carotenoid antioxidants – vitamins C and E (Pike et al. 2007b). However, female three-spined sticklebacks show a strong mating preference for

intensely red-coloured males (Bakker & Mundwiler 1994; Pike et al. 2007a), and the supplementation of vitamins C and E in that previous study had resulted in the males producing brighter throats, so it is likely that the females had based their mate preference on this visual cue alone (Pike et al. 2007b). As the supplementation of resveratrol in the present study did not increase the expression of the red nuptial colouration (Chapter 4), it is perhaps unsurprising that resveratrol did not influence female mate preference during the mate choice trials. The males in the present study had received a limited supply of carotenoids from early life, and based on previous work using this level of carotenoids, this was an inadequate supply to have fully invested in sexual ornamentation alongside allocating sufficient carotenoids to other competing functions (Pike et al. 2007c; Pike et al. 2010a). In fact, these low carotenoid males did not obtain as bright throats as males fed a diet high in carotenoids (See Figure 4.1, Chapter 4). Therefore, the possibility that the male three-spined sticklebacks supplemented with resveratrol had no opportunity to improve the expression of their nuptial colouration can be disregarded.

The present results also suggest that the females did not receive any alternative (and potentially important) mate cues mediated by resveratrol that were independent of the carotenoid-based signal. Perhaps resveratrol would increase sexual attractiveness in other species where ornament colouration is not a predominant cue in mate choice. Relevant to this might be the study by Arnold et al. (2010), who demonstrated a positive effects of dietary-derived colourless antioxidants (α-tocopherol, vitamin C and retinol) on sexual attractiveness in male budgerigars *Melopsittacus undulates* (Arnold et al. 2010), despite the fact that carotenoids do not play a role in plumage pigmentation in this species; Arnold et al. (2010) concluded that females were using some aspect of the males' phenotype or behaviour which was associated with physical fitness to choose between males.

Although resveratrol did not appear to play an important role in improving sexual attractiveness in the present study, it may still have a positive effect on reproductive success by influencing traits other than visual attractiveness, which were not considered in the present study. For instance, resveratrol has previously been shown to have positive effects on locomotor and cognitive performance in the turquoise killifish (Valenzano et al. 2006b). Therefore, perhaps if the males had been allowed to directly interact with each other during the mate choice trials then the resveratrol-fed males may have outcompeted males that had not been supplemented with resveratrol. In addition, resveratrol has been found to extend lifespan in both the turquoise killifish and a number of invertebrate species

including fruit flies and nematode worms (Howitz et al. 2003; Wood et al. 2004; Valenzano et al. 2006b). This effect on lifespan suggests that resveratrol may be able to positively influence reproductive potential through extending reproductive lifespan. However, this can only be speculated upon since survival to the next breeding season was not investigated in the present study as the subjects were culled for oxidative stress measurements immediately after their first breeding season. Lifespan is likely to be positively related to reproductive output in three-spined sticklebacks, as despite being short-lived, they can undergo multiple breeding attempts across a single breeding season and may also reproduce in more than one breeding season (Wootton 1976). For instance, it has been shown that lifespan is positively related to egg production in female three-spined sticklebacks (Lee et al. 2012). Also, lifespan in male three-spined sticklebacks was found to be positively correlated with the extent to which they maintained their nuptial throat colouration in their second breeding season (Lee et al. 2012).

5.5.2 Did male compensatory growth influence female mate preference?

In the present study, females did not distinguish between control males and males that had previously undergone a compensatory growth response after a period of poor food availability in early life. This finding is in accordance with the results of previous studies in both zebra finches and green swordtails which also demonstrated that females do not distinguish between control and compensated males (Blount et al. 2003a; Walling et al. 2007). Theoretical modelling has shown that animals with ornaments that undergo compensatory growth can disproportionately invest in their sexual ornament to strategically improve reproductive success (Lindström et al. 2005). Although the compensated males in the present study were able to fully compensate in terms of both body size and in their sexual ornament relative to controls, it may be that they adjusted their resource allocation strategy to minimise costs to their sexual attractiveness at the detriment of alternative traits. This has been demonstrated in zebra finches, where nestlings fed a low quality diet in early life were indistinguishable as adults in terms of both their body size and beak colouration after a period of growth compensation in comparison with continuously grown nestlings (Birkhead et al. 1999). However, that study illustrated how differential resource allocation can be costly as these compensated males suffered from a 30% higher mortality rate by 500 days of age in comparison with the males that had experienced continuous growth (Birkhead et al. 1999). The costs of a disproportionate investment in sexual ornamentation has also been demonstrated in three-spined

sticklebacks, where males with reduced carotenoid availability maintained their sexual ornament at the expensive of their reproductive output and lifespan (Pike et al. 2007a). Thus, although the compensated males in the present study did not have reduced sexual attractiveness in comparison with control males, it may be that maintaining this sexual ornament may have resulted in delayed costs later in life (Pike et al. 2007a).

5.5.3 Was female preference influenced by oxidative stress status in males fed a diet low in carotenoids?

In three-spined sticklebacks, the breeding season is energetically costly as the males must carry out highly energetic activities such as nest building, nest defence and fanning to provide the eggs with oxygen (Pike et al. 2007c). Therefore, males that are more susceptible to oxidative stress are less likely to provide a sufficient standard of parental care. This suggests that it would be particularly beneficial for females to be able to recognise males that were suffering from increased levels of oxidative stress. A previous study has shown that male three-spined sticklebacks fed on a low carotenoid diet were unable to increase their fanning activity during a period of hypoxia (Pike et al. 2007c), a time where males may be more susceptible to oxidative stress (Lushchak & Bagnyukova 2006). Therefore, being able to determine visual cues of oxidative stress status would be of paramount importance in the present study as these males had also been fed a limited diet of carotenoids.

The present study investigated whether three male oxidative stress measurements influenced female mate preference. There were no significant effects of a male's superoxide dismutase activity on his attractiveness to females. However, there was a female mate preference for males with a higher glutathione peroxidase activity. This suggests that there may have been deterioration in sexual attractiveness in males that had not been able to invest more in their endogenous antioxidant defences in terms of GPx. As the males did not differ in terms of their throat redness, it could be that females were using an alternative cue to assess the males oxidative stress status. Perhaps, the males that were unable to invest more in their endogenous defences were less active during the mate choice trials in terms of their courtship displays, since displays have been shown to be a physically tiring behaviour (Voituron et al. 2012) which is often a basis of mate choice in other species such as guppies and red jungle fowl *Gallus gallus* (Zuk et al. 1995; Kodric-Brown & Nicoletto 2001; Candolin 2003). However, as throat intensity was the only

attribute measured to investigate investment in sexual ornaments, it is also possible that other elements of the ornamentation such as size of throat or the pattern of colouration may have differed between males of different oxidative stress status.

5.5.4 Did a female's developmental and feeding history influence her "choosiness"?

The process of mate choice has been suggested to be costly in many species, since the assessment and comparison of potential mates is an energetic process which is likely to increase oxidative stress resulting from this increased physical activity (Pomianowski 1987; Byers et al. 2005; Toomey & McGraw 2012). Therefore, resveratrol may facilitate active choice in females through beneficial effects associated with its antioxidant properties (Fremont 2000; Gulcin 2010; Cai et al. 2011). Indeed, females supplemented with resveratrol in the present study spent significantly more time associating with males than females that had not been fed resveratrol. These findings support the recent work in captive house finches where females supplemented with a high carotenoid diet were more responsive during mate choice trials, a finding that was also attributed to the antioxidant properties of carotenoids (Toomey & McGraw 2012). Resveratrol may have had a positive effect on cognition which may alternatively explain why females spent more time associating with males in the mate choice trials. Indeed, the cognition results in Chapter 2 suggest that resveratrol plays an important role in neuroprotection. For example, it has been shown that resveratrol exhibits neuron protection in rodent models of neurodegeneration (Liu et al. 2011). Resveratrol has also been shown to retard neurodegeneration in *Nothobranchius furzeri* and enhance the clearance of degenerative neurons in *Nothobranchius guentheri* (Valenzano et al. 2006b; Genade & Lang 2013).

Females on a low carotenoid diet were found to spend more time associating with males during the mate choice trials if they had previously experienced *ad libitum* food rather than a previous period of food restriction. This result suggests that females that had undergone food restriction followed by compensatory growth were unable to cope with the costs of being choosy and were consequently more inactive during the mate choice trials and spent less time associating with the males. In support of this, male guppies have been found to reduce their courtship display rate when food availability is low (Kolluru & Grether 2008). In addition, female pronghorns *Antilocapra americana* are also found to reduce their mate search effort when their food resources have been low (Byers et al. 2006). However, this previous work did not identify compensatory growth as a restraint on active female mate

choice, but rather suggested that mate choice effort varied with current food availability (Byers et al. 2006).

5.5.5 Conclusions

In conclusion, this experiment demonstrated that neither compensatory growth nor the supplementation of a colourless antioxidant (resveratrol) influenced the attractiveness of male three-spined sticklebacks to breeding females, after controlling for size differences. However, growth compensation may have had negative functional consequences on a male's reproductive success if the males had been allowed to breed, and it would be interesting for future studies to investigate this and determine whether resveratrol supplementation mediates any of these potential differences. Most interestingly, females supplemented with resveratrol in the present study spent significantly more time associating with males than females that had not been fed resveratrol. However, further research is required into the role of dietary-derived colourless antioxidants in sexual attractiveness as the results remain inconclusive. Perhaps more interestingly, their physiological roles in mediating other potential fitness traits associated with reproductive success should be the focus of more attention.

CHAPTER 6 – OXIDATIVE STRESS, DIETARY ANTIOXIDANTS AND COGNITIVE FUNCTION IN THREE-SPINED STICKLEBACKS

6.1 ABSTRACT

Oxidative stress has been suggested to play a causative role in age-related cognitive decline. Therefore, in order to maintain cognitive function it is crucial that oxidative damage is reduced. Resveratrol has been reported to have protective abilities which are able to impede cognitive ageing which have been mostly attributed to its neuroprotective and antioxidant properties. Dietary supplementation studies often assess exploratory activity as an indicator of the effects of nutrition on cognitive function. This study utilised the open field test in to investigate whether specific dietary supplements can decelerate this cognitive decline with age. A sex specific trade-off was demonstrated whereby only the females that had exhibited compensatory growth incurred a significant reduction in their exploratory drive with age. The results produced conflicting perspectives on the abilities of resveratrol in reducing anxiety-related behaviour. Additionally, carotenoids did not have a significant effect on any of the four markers measured to infer rate of cognitive ageing.

6.2 INTRODUCTION

6.2.1 Oxidative stress, dietary antioxidants and cognitive function

Age-related cognitive decline is accepted as a widespread and inherent feature of the "normal" process of ageing, which occurs even in the absence of disease (Salthouse 1991; Moscovitch & Winocur 1995). There is increasing evidence to suggest that oxidative stress plays a causative role in age-related cognitive decline (Dröge & Schipper 2007; Glade 2010). The brain is a continuously-operating and high energy-demanding organ which produces large amounts of free radicals (Reiter 1995; Floyd 1999). The amount of free radicals produced in the brain increases with the complexity of the cognitive processes being undertaken (Loft et al. 1994). Therefore, upholding constant oxidative balance is crucial in the maintenance of cognitive function (Glade 2010).

The brain is a site of high metabolic activity which can often generate an imbalance between the production of local free radicals and the availability of antioxidants to counteract the oxidative stress assault (Davies 2000; Luo 2006). Cognitive function can

therefore become impaired as this imbalance results in the oxidative modification of important components of the brain such as proteins, lipids and nucleic acids that play a crucial role in supporting its healthy functioning (Head 2009; Bishop et al. 2010). As a result, there has been a wealth of research into the use of nutritional enrichment interventions to preserve cognitive function by means of their protective antioxidant abilities (Carrié et al. 2000; Kang & Grodstein 2008; Dal-pan et al. 2011). For instance, chronic coffee and caffeine ingestion in male Wistar rats, *Rattus norvegicus* was found to improve cognitive function in an object recognition task used to assess long-term memory (Abreu et al. 2011). This was indicated to be through their protection of the cerebral antioxidant system (Abreu et al. 2011). Additionally, Fischer rats fed a diet supplemented with vitamin E from adulthood to middle age exhibited significantly better performance in a Morris water maze task which tested spatial learning and memory (Joseph et al. 1998). There are numerous other examples of nutritional supplements, including an array of vitamins and fatty acids, that have been shown to exhibit protective effects on cognitive function with age (Arzi et al. 2004; Mazereeuw et al. 2012). However, the polyphenol resveratrol has received an unprecedented amount of attention and research in this field (Robb & Stuart 2010).

6.2.2 Resveratrol and its proposed protective effects on cognitive ageing

The protective abilities of resveratrol in impeding cognitive ageing have been documented in numerous studies over the last decade (Vang et al. 2011). More specifically, resveratrol's protective abilities in delaying cognitive ageing have been mostly attributed to its neuroprotective and antioxidant properties (Fukui et al. 2010). For instance, resveratrol has been found to protect against oxidative neuronal death in a mouse hippocampal neuronal cell line (Fukui et al. 2010). This was accredited to its ability to selectively induce the expression of an endogenous antioxidant enzyme, mitochondrial superoxide dismutase (MnSOD), which was able to reduce oxidative stress in order to protect the neuronal cells from an experimentally-applied neurotoxic exposure (Fukui et al. 2010). Additionally, in two independent experiments, resveratrol demonstrated neuroprotection in the brains of healthy male Wistar rats by dose-dependently reducing oxidative damage, increasing antioxidant enzyme activity and up-regulating important detoxifying iron proteins (Mokni et al. 2007; Sebai et al. 2009). However, evidence suggests that resveratrol has poor bioavailability *in vivo*, so poor in fact that it is unlikely that its protective abilities in cognitive ageing can be credited to direct chemical

antioxidant effects (Wenzel & Samoza 2005; Robb & Stuart 2010). Rather, resveratrol's protective effects on cognitive ageing are more likely to be attributed to its abilities to indirectly reduce oxidative stress via the upregulation of endogenous antioxidant enzymes such as MnSOD (Robb & Stuart 2010).

Nevertheless, despite a lack of understanding of the underlying mechanisms involved, resveratrol's protective effects on cognitive ageing have also been demonstrated *in vivo* in a few behavioural studies (Valenzano et al. 2006b; Harada et al. 2011; Yu & Li 2012). For example, in adult male grey mouse lemurs *Microcebus mirinus,* an 18-month oral resveratrol supplement improved performance in two tests used to evaluate cognitive performance (Dal-pan et al. 2011). Resveratrol supplementation reduced the number of errors made by the lemurs in a spatial memory test and improved performance by 19% in a spontaneous alternation task, in comparison with controls (Dal-pan et al. 2011). However, it should be highlighted that resveratrol did not improve performance in a number of other alternative cognition and motor tasks carried out in the same study, implying restrictions in the generality of its enhancing abilities (Dal-pan et al. 2011). Resveratrol was also ineffective in improving performance in a radial arm maze task in five-month-old male and female mice fed with resveratrol for an eight-week period (Chang et al. 2012). Additionally, within the same study, mice fed an identical dose of an alternative antioxidant, pterostilbene, did show significant improvements in performance in the same radial arm maze task suggesting that pterostilbene is a more potent modulator of cognitive function in terms of spatial and memory learning (Chang et al. 2012). Moreover, a recent study (Friedman et al. 2013) found no support for the neuroprotective abilities of resveratrol. In this study, young male and female Sprague-Dawley rat pups were treated with kainic acid to induce seizures and resveratrol failed to reduce injuries in the brains of the treated mice.

Although the studies finding no effect of resveratrol as a neuroprotective agent are relatively few and far between (Chang et al. 2012; Friedman et al. 2013), caution should be made, as the neuroprotective effects of resveratrol to date have been predominantly restricted to rodent species and neuronal cell lines (Robb & Stuart 2010). The few other taxa that have been investigated include two closely related species of fish, *Nothobranchius furzeri* and *Nothobranchius guentheri* (Valenzano et al. 2006b; Yu & Li 2012).

There has also been a disproportionate focus on the effects of resveratrol in protecting against injury and age-related disease, with very few studies investigating its effects on "normal" cognitive ageing (Genade & Lang 2013). For example, resveratrol was found to reduce oxidative stress and display neuroprotection after an ischemia-reperfusion injury in male New Zealand white rabbits *Oryctolagus cuniculus* (Kiziltepe et al. 2004). Epidemiological studies such as Kiziltepe et al. (2004) are predominantly focused on resveratrol's potential clinical use in disease treatments with a lack of consideration of the mechanisms underlying its ability to reduce the rate of cognitive ageing. For instance, many of these studies focus on resveratrol's specific use in the prevention of neurodegenerative pathologies such as Parkinson's and Alzheimer's diseases (Richard et al. 2011). In addition, many of these rodent studies investigate the neuroprotective properties of resveratrol on male subjects only, and the exposure to resveratrol is often in simultaneous combination with other antioxidants (Kumar et al. 2007; Bist & Bhatt 2010; Singleton et al. 2010). It has been highlighted that there is currently a lack of sufficient evidence to quantify resveratrol's robustness as a neuroprotectant and that more studies are required to evaluate any potential species-specific effects resveratrol may have (Yu & Li 2012).

6.2.3 Utilising the paradigm of open field tests to assess exploratory activity as an age-related marker

It has been shown across a number of species that exploratory activity declines with age in terms of an animal's tendency and ability to explore new areas and novel objects, supporting its applicability as an effective indicator of cognitive ageing (Ingram 2000; Siwak et al. 2001; Rodenburg et al. 2003; Rosado et al. 2012). For instance, a significant decline in several indices of exploration and general activity were found in rats of 24 months of age when compared with their first assessment at one month of age (Glenn et al. 2008). Dietary supplementation studies often assess exploratory activity as an indicator of the effects of nutrition on cognitive function (Coluccia et al. 2009; Abreu et al. 2011). In addition, a large majority of these studies have utilised the open field test in particular to demonstrate age-dependent decline in cognitive performance, simultaneously investigating whether specific dietary supplements can decelerate this decline (Valenzano et al. 2006b; Glenn et al. 2008; Pietrelli et al. 2012). For example, the open field test was used to assess exploratory behaviour in relation to age and supplementation of dietary omega-3 fatty acids in female mouse lemurs *Microcebus murinus,* where an age-related decline in

exploratory activity was demonstrated by an increase in latency to move from the centre of an open field in the aged lemurs (Languille et al. 2012). However, contrary to what was expected, the supplementation of omega-3 fatty acids in the aged lemurs resulted in a decrease in exploratory activity in terms of both latency to move and also distance travelled in the open field (Languille et al. 2012). These findings led this particular study to conclude that dietary supplementations provided from a stage earlier in life may be more effective in reducing cognitive decline with age (Languille et al. 2012).

Open field exploration is a standard behavioural test originally created by Hall in the 1930's to assay exploratory movements and anxiety in rodents (Hall & Ballachey 1932; Hall 1934). More recently, the open field test has become commonly used in behavioural studies in fish species, such as the lion-headed cichlid *Steatocranus casuarius,* the turquoise killifish *Nothobranchius furzeri* and the paradise fish *Macropodus opercularis* (Gerlai & Csányi 1988; Budeav et al. 1999; Valenzano et al. 2006a). The open field test quantifies the exploration of experimental subjects to a novel environment using behavioural measurements such as the distance moved and the proportion of time spent moving (Gould et al. 2009). A decreased exploratory activity is often considered to represent increased anxiety in the open field paradigm (Sönmez et al. 2007). Thigmotaxis, a preference to avoid the centre of a novel environment and remain close to its walls, is an anxiety-related behaviour often measured in the open field arena (Schnörr et al. 2012; Norton 2012). Age-related reduction of exploratory activity in an open field paradigm has been previously quantified in *Nothobranchius furzeri,* where a reduction in time spent moving was found at nine-weeks old when compared with five-week old fish (Valenzano et al. 2006b).

The aim of the present study was to investigate how compensatory growth affected later-life cognitive function in three-spined sticklebacks in terms of exploratory activity and anxiety-related behaviour in an open field test. Compensatory growth has been found to impair later life cognitive performance in zebra finches *Taeniopygia guttata*: the magnitude of compensatory growth exhibited by the birds was found to negatively affect subsequent learning performance in a relatively simple cognitive task (Fisher et al. 2006). While the mechanism underlying that effect was not investigated, it is possible that it was related to oxidative damage since compensatory growth may cause an increase in levels of oxidative stress (See Section 3.2.2, Chapter 3). In the present study I also therefore examined whether dietary supplements of resveratrol and two carotenoids, lutein and

astaxanthin, reduced the expected age-dependent decline in cognitive performance and whether this was influenced by an earlier period of compensatory growth.

6.3 METHODS

6.3.1 Source of fish and rearing conditions

Underyearling three-spined sticklebacks were collected between the 1st and 6th December 2011, under the procedures described in Chapter 2. Following capture, the fish were transported to the University of Glasgow and transferred to acclimatisation aquaria (45-L and density 3 fish L^{-1}) for eight weeks and fed *ad libitum* (i.e. 10% of body mass/day) with frozen chironomid larvae until the 24th February 2012 when the feeding treatments commenced.

On the 4th February 2012, all 120 fish were anaesthetised and measured for standard length ($\pm$0.01 mm) and wet mass ($\pm$0.001 g). All fish were then randomly assigned into groups of 3 and re-housed in smaller (7-L) tanks (33 × 18 × 19 cm). Individuals could then be identified for the remainder of the experiment on the basis of their size. The fish were kept under the husbandry procedures detailed in Chapter 2.

6.3.2 Growth and dietary supplement manipulations

The 120 fish were allocated to 8 feeding treatments, which are described in Table 2.1 in Chapter 2, with the exception that resveratrol was supplemented in the present study at a greater concentration of 600μg g^{-1} of food. In a previous turquoise killifish study, an identical concentration was the maximum dose tested which was found to significantly increase median and maximum lifespan (Valenzano et al. 2006b). As supplementation of resveratrol was shown to dose dependently increase lifespan (Valenzano et al. 2006b), the present study adopted this concentration in order to assess its effectiveness in reducing age-dependent cognitive decline. The feeding treatments began on 24th February 2012. Half of the fish (i.e. 20 tanks of 3 fish) were randomly assigned to the compensatory (C) growth regime, and the remainder to the *ad libitum* (A) growth regime. The restricted food ration was applied for a four-week period (Period 1); by this time point the C growth regime fish were significantly smaller in size in comparison with the A growth regime fish.

This restricted ration was then uplifted on 26th March 2012 to induce compensatory growth in the C regime fish (Period 2).

The 20 tanks within both the C and A growth regimes were further randomly assigned into 5 replicate tanks for each of the four dietary supplement manipulations. These dietary supplement manipulations are described in Section 2.3.3 in Chapter 2. Thus, overall within this experiment there were 8 feeding treatments (2 growth regimes × 2 resveratrol regimes × 2 carotenoid regimes, each with 5 replicate tanks containing 3 fish), allowing evaluation of the effect of dietary restriction-induced compensatory growth on later-life performance in an open-field test in mature sticklebacks (Figure 6.1). In addition, it could also be determined whether dietary supplements of resveratrol and carotenoids influenced their response. On 18th June 2012, the fish were sexed on the basis of their colouration, where males were identified by their reddish throat.

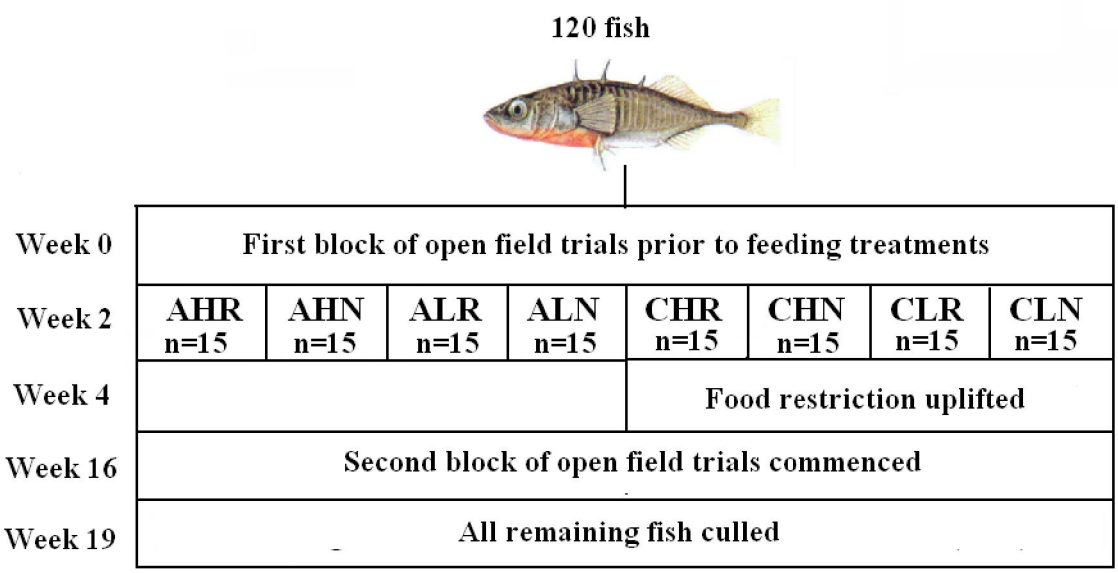


Figure 6.1 Schematic of experimental design. At week 0, 120 fish commenced the first block of open field trial. At week 2, these fish were then equally divided into the eight feeding treatments described in (Table 2.1, Chapter 2). At week 4 the food restriction was uplifted in the four compensatory growth regime fish (CHR, CHN, CLR and CLN). At week 16, the same fish were re-measured in a second block of open field trials. At week 19, all remaining fish were culled.

6.3.3 Apparatus and behavioural testing in an open field tank

Behavioural testing was performed using a rectangular tank (length 60cm × width 39cm × height 42cm). The open field tank dimensions were selected after researching the open field tank measurements used in previous fish studies (Valenzano et al. 2006b; Archard &

Braithewaite 2010). Therefore, the tank can be confidently regarded as large enough in relation to the size of the sticklebacks to infer exploratory tendencies. The tank was filled to a level of 10cm with 50% fresh water and 50% of water taken from the home tank of the experimental fish. A water change was carried out after every trial to eliminate any potential sensory cues left in the water from previous fish, which may have otherwise affected behaviour. The open field behaviour trials were performed in the same room as the home tanks in order to reduce any confounding effects of lighting and temperature on behaviour. In addition, the experimenter was not present during the recording periods in order to prevent disturbance to the fish. As described in Chapter 2, the temperature and the photoperiod had been adjusted to match the mean ambient conditions at the source river for that time of year. The photoperiod was achieved using fluorescent lighting and controlled by electronic timers. Individual change in exploratory performance was quantified by conducting the open field trials once in early life - prior to the implementation of the feeding treatments when the fish were approximately 8 months old - and then once again later in life when the fish had received their allocated feeding treatments for 15 weeks and had become sexually mature. The first and second block of open field trials began the week beginning the 13th February 2012 (week 0) and the 18th June 2012 (week 16), respectively.

Prior to testing, each fish was placed into an opaque 80mm-diameter acclimation tube, which was placed directly into the centre of the open field tank (shown in Fig. 6.2) and left to acclimatize for 2 minutes. Each trial consisted of a five-minute recording period, which was consistent to the duration commonly used in other open field tests (Gould et al. 2009; Cachat et al. 2011; Godwin et al. 2012). To begin each trial, the acclimatisation tube was lifted and removed to allow the fish to escape, and the subsequent movements of the fish were filmed directly from above using a tripod-mounted digital video camera recorder (SONY DCR-SX15E) at a fixed distance of 110cm.

6.3.4 Post-trial video analysis

The exploratory behaviour of the fish was quantified in four different ways. The extent of exploration of the test tank was measured by outlining a 3×3 matrix of rectangles onto an acetate sheet and superimposing this onto the computer monitor during the video analysis procedure (Figure 6.2). The grid was not marked onto the base of the tank in order to prevent any potential confounding effects the markings may have had on influencing behaviour during the trials. Both the total number of zone borders crossed by the fish and

the number of zones entered out of a total of nine were recorded manually from the video recordings and were then used as two measures indicative of exploratory behaviour.

The third measure was of thigmotaxis. In order to measure this, the video analysis procedure was repeated for all video trial recordings using an alternative acetate sheet which outlined a 27mm boundary used to assess thigmotaxis, measured as the total proportion of time the fish spent along the walls of the open field tank as opposed to time spent within the centre of the tank (Figure 6.3). The criterion for the width of the wall zone was based on measurements used in an open field test using Mexican blind cavefish *Astayanax* sp., where wall preference behaviour was characterized as when the fish were less than 0.5 standard lengths away from the perimeter of the wall of the open tank (Sharma et al. 2009). In the present study, this was based on the mean length of the fish across all treatments at the time of testing of the second block of open field trials.

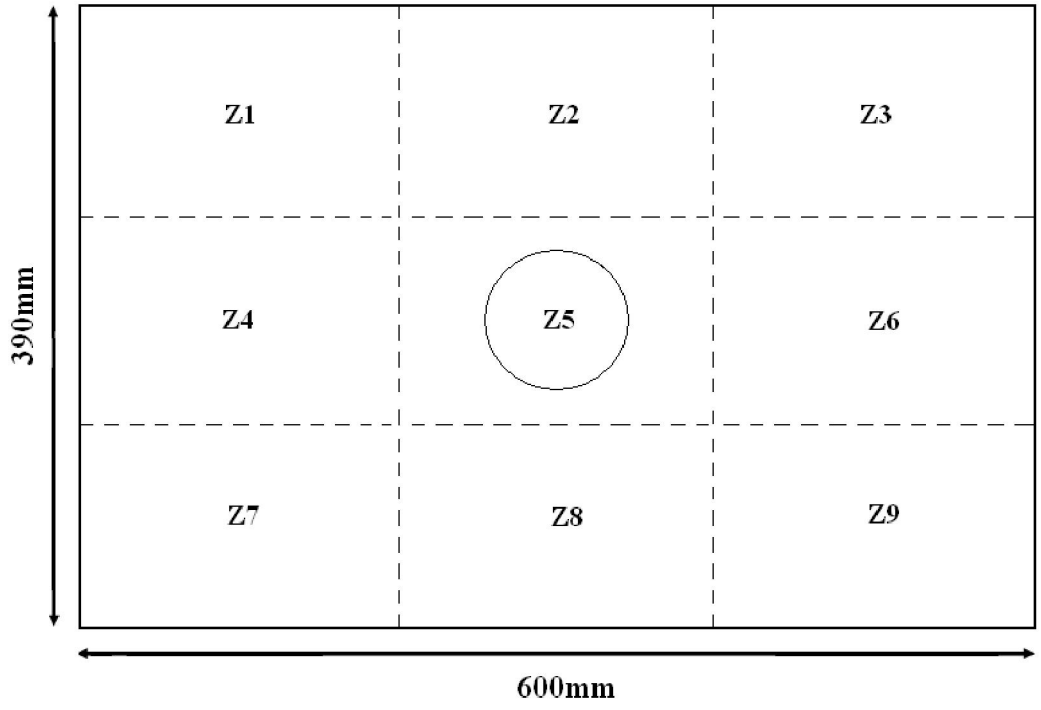


Figure 6.2 Schematic drawing of the novel arena tank used for the open field test with the superimposed 3×3 matrix of squares used to measure both the total number of zone borders crossed by the fish alongside the total number of zones entered out of nine. The sphere indicates the central position of the acclimatisation tube.

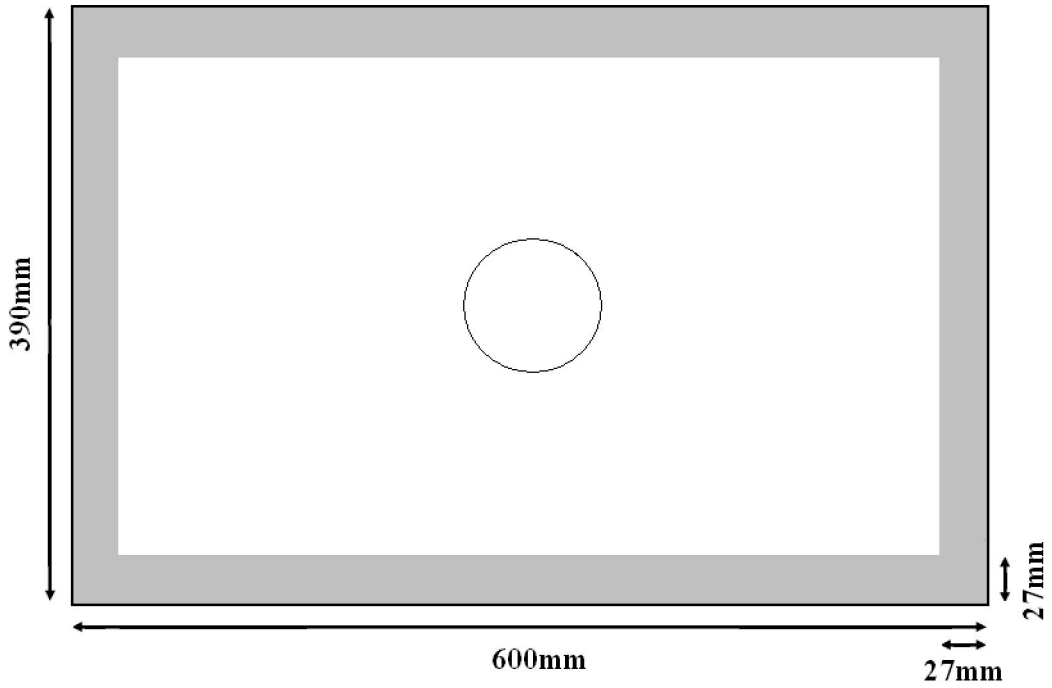


Figure 6.3 Schematic drawing of the novel arena tank used for the open field test with the superimposed 27mm border wall boundary used to assess thigmotaxis measured as proportion of time spent along wall. The sphere indicates the central position of the acclimatisation tube.

The final measure was latency to begin exploration of the open field tank. The video analysis procedure was then repeated for a final time using another alternative acetate sheet, which outlined a 69mm^2 square-shaped area, which marked the boundary of each experimental animal's starting position, centred on the acclimatization tube. The latency to leave this starting position was measured as the number of seconds it took for the whole body of the fish to depart the square-shaped boundary area.

6.3.5 Statistical analysis of growth

The fish were measured at five regular intervals across the experiment, twice at the beginning of the experiment before growth manipulation commenced, once during the growth manipulation period (Period 1) and twice after the restricted ration had been uplifted (Period 2). Regular measurements made it possible to identify each fish within the group of three held in a tank and so determine its growth pattern. Therefore, treatment effects on growth patterns were assessed by comparing the standard body length and mass of each individual fish from each tank at each sampling point. In order to prevent bias in the experimental results due to any size-selective mortality, these analyses of growth were based on the 91 fish (50 females and 41 males) that survived the duration of the

experiment. Differences in standard body length and mass between the *ad libitum* and compensatory growth regimes were tested using a multivariate analysis of variance (MANOVA), as were differences in standard body length and mass between all eight feeding treatments.

6.3.6 Statistical analyses of open field behaviours

The effects of growth regime and dietary supplementation on the change in open field behaviour over the experimental period were analysed separately for the four measures of behaviour (total number of zones explored out of nine, number of zone borders crossed, proportion of time spent within the wall zone and latency to leave starting point) using general linear mixed models (GLMMs), with growth regime (A or C), carotenoid supplementation (H or L), resveratrol supplementation (R or N) and sex as fixed effects, body length at first recording and body length at second recording as covariates and tank number as a random factor, plus all two-way interactions among variables. Non-significant variables were sequentially dropped from the analysis.

All means are described with standard errors and all analyses in this present chapter were carried out using R (R Core Development Team, version 2.15.0).

6.4 RESULTS

6.4.1 Compensatory growth response after food restriction

At the start of the experiment, there were no differences in either the standard length or mass between the two growth regime groups (A and C) (MANOVA: Wilk's $\lambda = 0.954$, $F_{2,88} = 2.109$, $p = 0.128$; Figure 6.4), or among the eight feeding treatments overall (see Table 2.1 in Chapter 2 methods for description of treatment groups) (MANOVA: Wilk's $\lambda = 0.812$, $F_{14,164} = 1.290$, $p = 0.219$). However, after the 4-week manipulation of growth regimes (end of Period 1) there were significant differences in both standard length and mass, between the two growth regime groups (A and C, pooling data for treatments differing in dietary supplements) (MANOVA: Wilk's $\lambda = 0.901$, $F_{2,88} = 4.843$, $p = 0.010$). At this time point, the C fish were 5.9% shorter (ANOVA, $F_{1,90} = 7.053$, $p = 0.009$) and 19.0% lighter (ANOVA, $F_{1,90} = 9.344$, $p = 0.003$) than A fish.

There were no effects of resveratrol supplementation within the A growth regime (MANOVA: Wilk's λ= 0.952, $F_{2,45}$ = 1.126, p = 0.333 or within the C growth regime: Wilk's λ = 0.891, $F_{2,40}$ = 2.437, p = 0.100. There were also no effects of carotenoid supplementation at the end of Period 1 within the A growth regime: Wilk's λ = 0.969, $F_{2,45}$ = 0.712, p = 0.496 or within the C growth regime: Wilk's λ = 0.956, $F_{2,40}$ = 0.929, p = 0.403.

The differences in size began to disappear once the C growth regime fish were transferred onto the A growth regime diet, and after 4 weeks full compensation had occurred as there were no longer significant differences in size between the two growth regime groups (A and C) (MANOVA: Wilk's λ = 0.938, $F_{2,88}$ = 2.892, p = 0.06) (Figure 6.4) or among the eight feeding treatments overall (MANOVA: Wilk's λ = 0.833, $F_{14,164}$ = 1.117, p = 0.35).

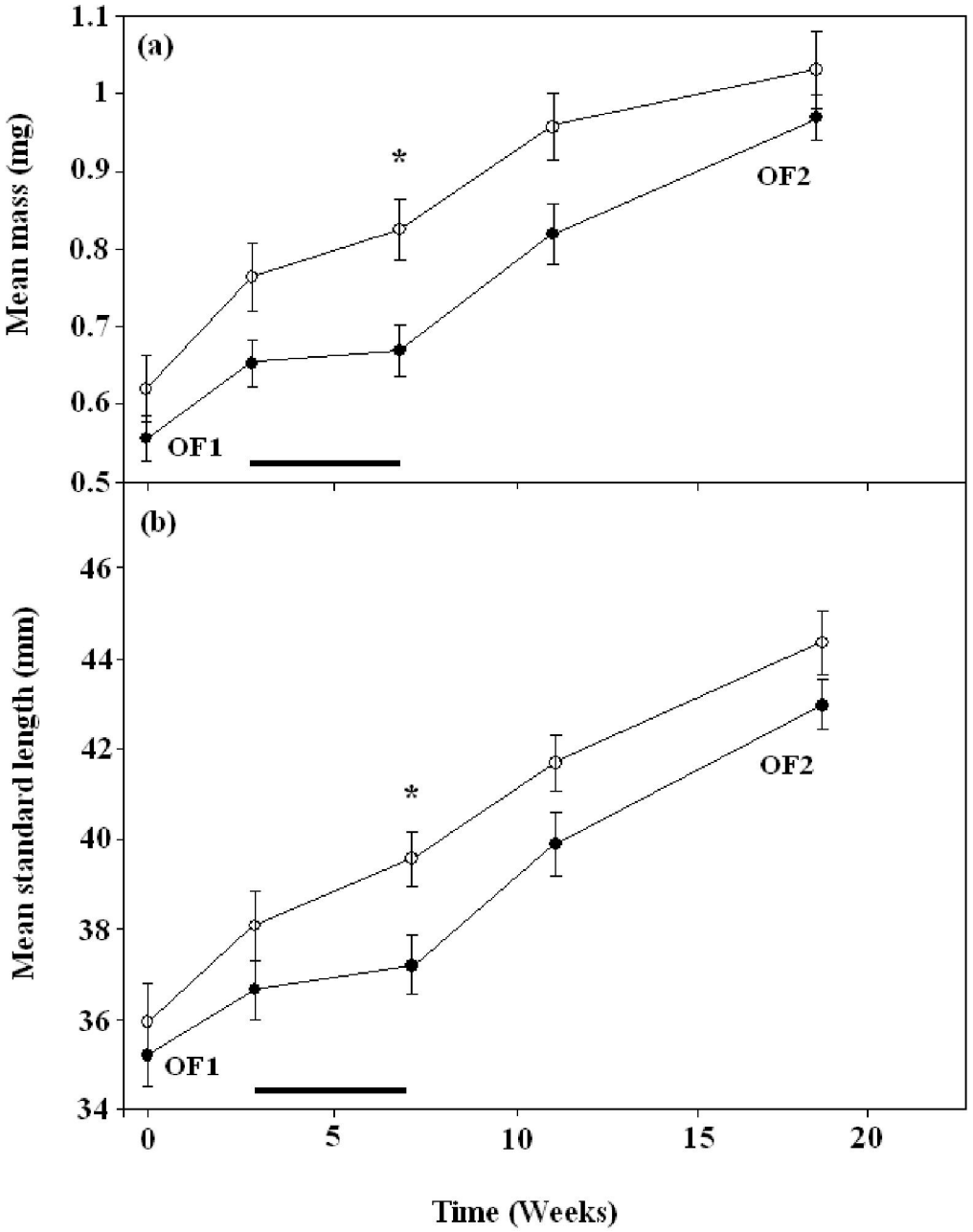


Figure 6.4 Growth trajectories (mean ± s.e.) for (a) mass and (b) standard length of three-spined sticklebacks in relation to growth regime (*ad libitum*, open circles; compensatory growth, closed circles). The thick horizontal line indicates the period of growth manipulation (4 weeks), after which point all fish were fed *ad libitum* but were still maintained on their original dietary supplementations. The asterisk indicates a significant difference in mass and length between the A and C growth regime groups ($p<0.05$); n = 91 fish. "OF1" and "OF2" indicate the timing of the first and second block of open field trials, respectively. The experiment concluded at week 19 when the fish were culled after their final growth measurements.

6.4.2 Zone border crossings

A significant interaction was found between sex and resveratrol supplementation with respect to the change in activity patterns over the two trials (GLMM, t_{49} = -2.272, p = 0.028) (Table 6.1, Figure 6.5). Males that had not received resveratrol showed a significantly larger increase in the number of zone border crossings between the first and second block of open field trials (mean ± s.e. = 12.35 ± 3.97), in comparison with the males that had received resveratrol (mean ± s.e. = -0.92 ± 3.66, t-test, t = -2.416, p = 0.02) (Figure 6.5). There was no significant difference between the resveratrol fed females (mean ± s.e. = 2.19 ± 3.18) and the females not fed resveratrol (mean ± s.e. = -1.17 ± 3.62, t-test, t = 0.698, p = 0.49) in terms of their change with age in zone border crossings (Figure 6.5).

Table 6.1 Results of a general linear mixed model examining age-related change in the total number of zone border crossings undertaken in relation to growth regime, sex, carotenoid supplementation and resveratrol supplementation. Rearing tank was included as random factor.

Final model	Estimate	SE	DF	*t*	*p*
Resveratrol (Y)	3.359	4.873	38	0.689	0.50
Sex (M)	13.520	5.458	49	2.477	0.017
Regime (A)	2.711	3.688	36	0.735	0.47
Carotenoid (L)	3.639	3.667	37	0.992	0.33
Resveratrol (Y) × Sex (M)	-16.629	7.317	49	-2.272	0.028

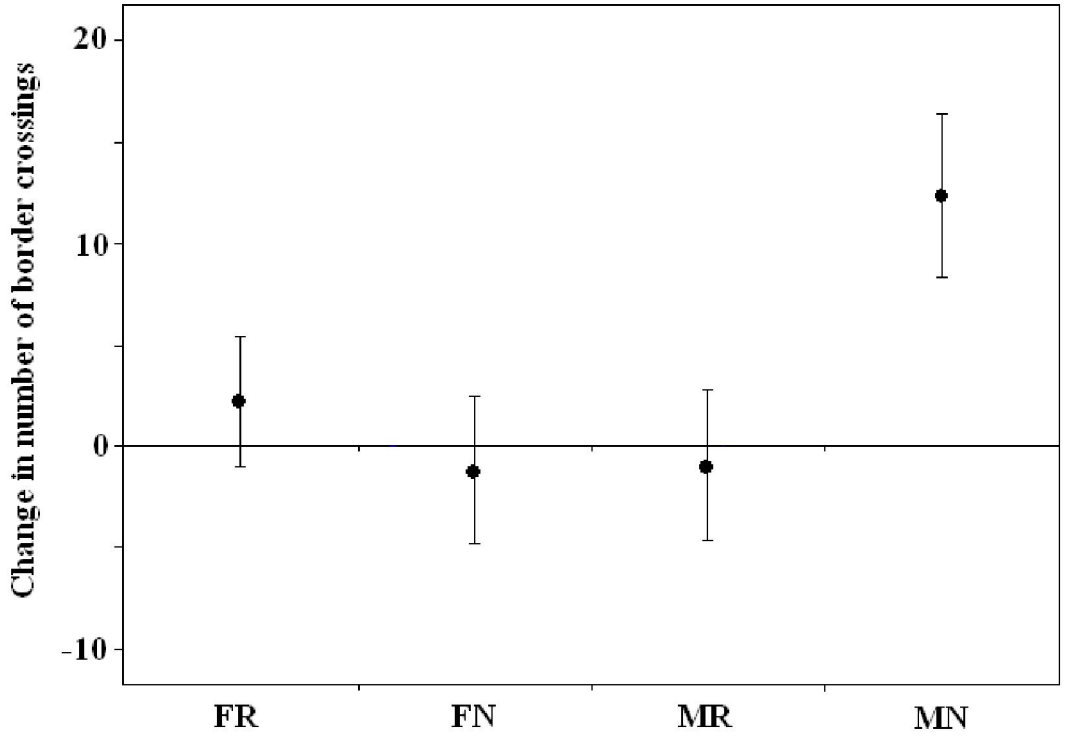


Figure 6.5 Mean ± s.e. Age-related change in the number of zone border crossings over the experimental period for male (M) or female (F) fish fed either a diet with resveratrol (R) or without resveratrol (N).

6.4.3 Number of zones explored

When analysing the change in the overall area explored in the first and second trial, a significant interaction was found between sex and whether fish were fed on a compensatory growth or an *ad libitum* growth regime (GLMM, t_{48} = -2.573, p = 0.013) (Table 6.2). There was no difference between males fed on the *ad libitum* growth regime (mean ± s.e. = 0 ± 0.872) and the compensatory growth regime (mean ± s.e. = 1.217 ± 0.832, t-test, t = 0.981, p = 0.33) in terms of their age-related change in the number of zones explored (Figure 6.6). In contrast, females that were fed on the compensatory growth regime significantly reduced their exploratory activity between the first and second block of open field trials (mean ± s.e. = -1.350 ± 0.799), in comparison with the females that were fed on the *ad libitum* growth regime (mean ± s.e. = 1.667 ± 0.690, t-test, t = -2.837, p = 0.007) (Figure 6.6).

Independent of these effects of the compensatory growth regime, a significant interaction was found between sex and whether fish were fed a diet supplemented with resveratrol or not (GLMM, t_{48} = -2.114, p = 0.039) (Table 6.2). There was no difference between females

fed resveratrol (mean ± s.e. = 0.615 ± 0.545) and not fed resveratrol (mean ± s.e. = 0.292 ± 0.755, t-test, t = 0.286, p = 0.78) in terms of their age-related change in the number of zones explored (Figure 6.7). In contrast, males that were not fed resveratrol significantly increased their exploratory activity between the first and second block of open field trials (mean ± s.e. = -0.542 ± 0.710), in comparison with the males that were fed resveratrol (mean ± s.e. = 2.412 ± 0.978, t-test, t = -2.444, p = 0.020) (Figure 6.6).

Table 6.2 Results of a general linear mixed model examining age-related change in the total number of zones explored in relation to growth regime, sex, carotenoid supplementation and resveratrol supplementation. Tank was included as random factor.

Final model	Estimate	SE	DF	*t*	*p*
Resveratrol (Y)	0.470	1.063	37	0.442	0.660
Regime (A)	3.040	1.083	37	2.806	0.008
Carotenoid (L)	-0.024	0.810	36	-0.030	0.977
Sex (M)	4.467	1.452	48	3.077	0.004
Resveratrol (Y) × Sex (M)	-3.373	1.595	48	-2.115	0.039
Regime (A) × Sex (M)	-4.124	1.603	48	-2.573	0.013

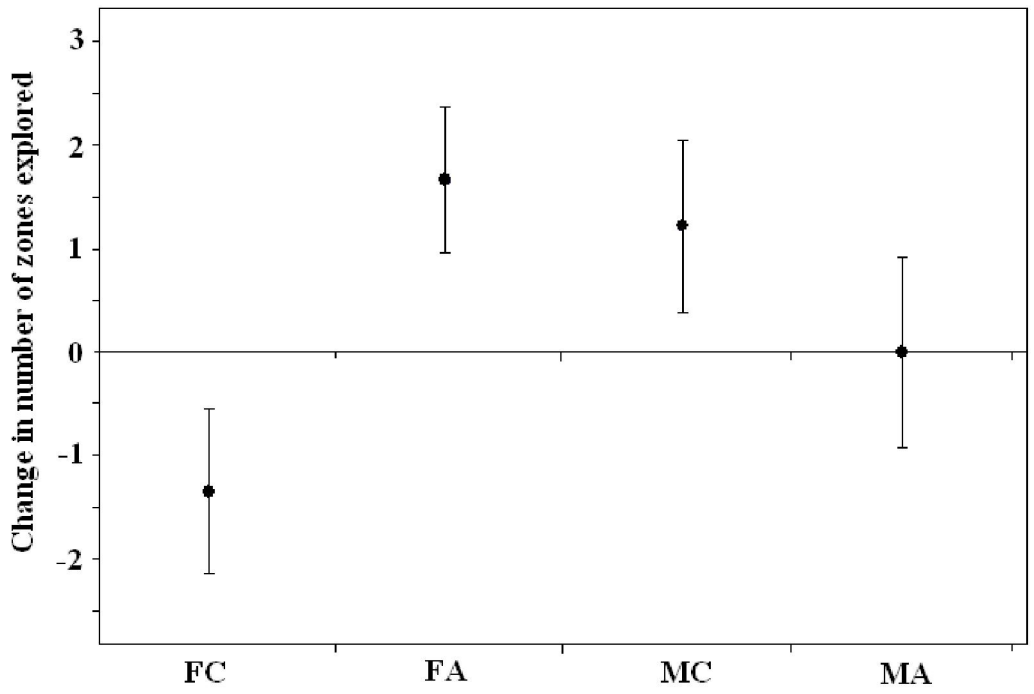


Figure 6.6 Mean ± s.e. Age-related change in the number of zones explored over the experimental period for male (M) or female (F) fish fed either a compensatory (C) or an *ad libitum* growth regime (A).

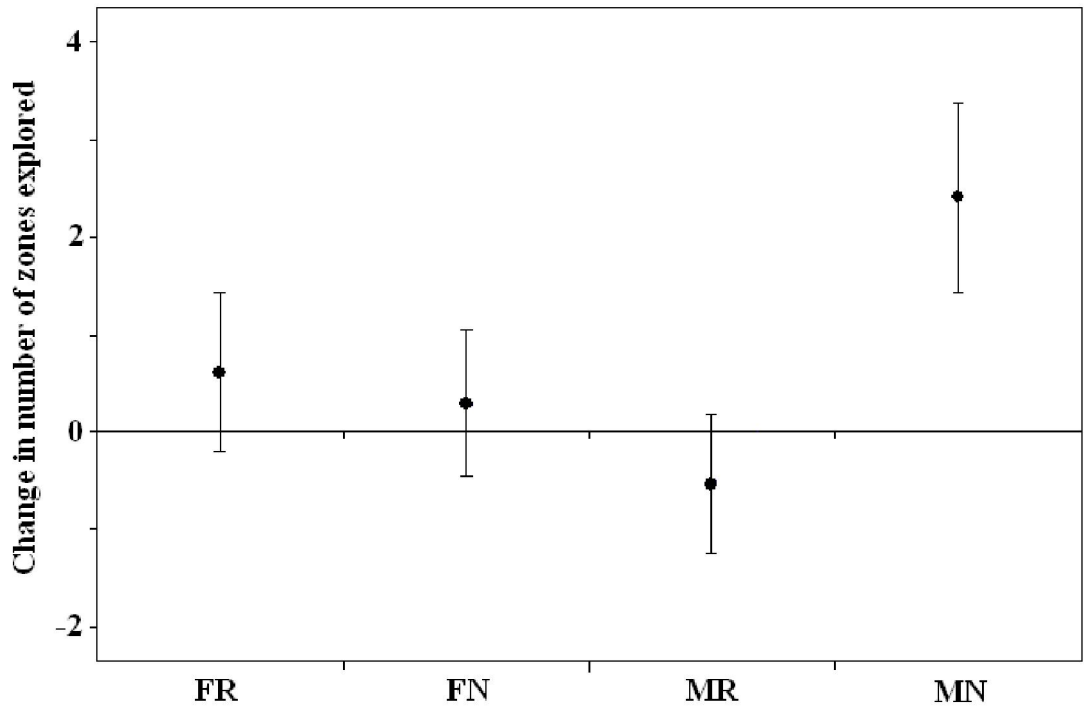


Figure 6.7 Mean ± s.e. Age-related change in the number of zones explored over the experimental period for male (M) or female (F) fish fed either a diet with resveratrol (R) or without resveratrol (N).

6.4.4 Proportion of time spent near wall

The analysis of thigmotaxis revealed a significant interaction between growth regime and whether fish were fed a diet supplemented with resveratrol or not (GLMM, $t_{36} = 3.600$, $p = 0.001$) (Table 6.3). However, the proportion of time spent within the wall zone of the open field tank between the first and second block of open field trials (tested 16 weeks apart), was not significantly different between the *ad libitum* growth regime fish that had received resveratrol (mean ± s.e. = -0.173 ± 0.097) and the fish that had not received resveratrol (mean ± s.e. = 0.079 ± 0.103, t-test, $t = -1.779$, $p = 0.08$) (Figure 6.8). In addition, there was no difference between fish on a compensatory growth regime that had been fed resveratrol (mean ± s.e. = -0.005 ± 0.109) and not fed resveratrol (mean ± s.e. = -0.187 ± 0.123, t-test, $t = -1.788$, $p = 0.08$) in terms of their age-related change in thigmotaxis (Figure 6.8).

Table 6.3 Results of a general linear mixed model examining age-related change in the total time spent within wall zone in relation to growth regime, sex, carotenoid supplementation and resveratrol supplementation. Tank was included as random factor.

Final model	Estimate	SE	DF	*t*	*p*
Resveratrol (Y)	- 105.09	44.19	36	-2.378	0.023
Regime (A)	-141.50	45.85	36	-3.086	0.004
Carotenoid (L)	-16.765	30.87	35	-0.543	0.590
Sex (M)	-15.207	31.50	48	-0.483	0.631
Length in June	11.06	3.55	50	3.120	0.003
Resveratrol (Y) × Regime (A)	219.70	61.03	36	3.600	0.001

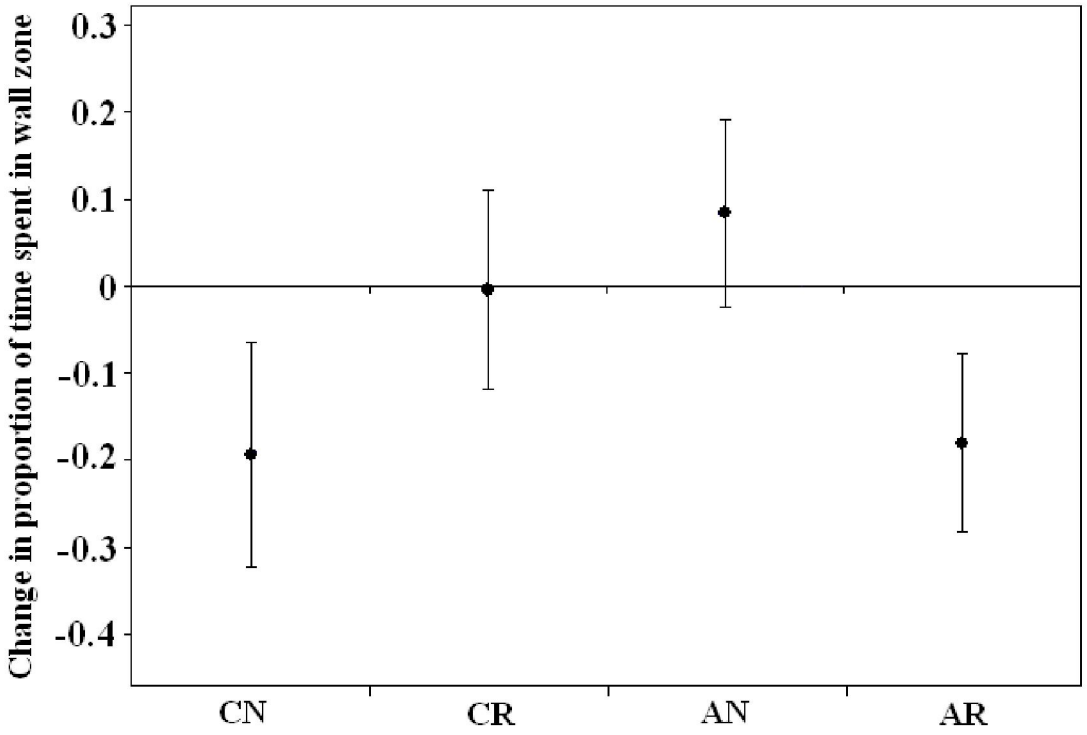


Figure 6.8 Mean ± s.e. Change in the proportion of time spent within the wall zone over the experimental period for fish fed either a diet with resveratrol (R) or without resveratrol (N) and fed either a compensatory (C) or an *ad libitum* (A) growth regime.

6.4.5 Latency to leave starting position

There was no difference in the change in latency to depart the starting position of the open field tank, in relation to growth regime, whether resveratrol was present or absent in the

diet, or whether carotenoids were fed in a high or low dose in the diet (Table 6.4, Figure 6.9).

Table 6.4 Results of a general linear mixed model examining age-related change in the latency to depart the starting position of the open field tank in relation to growth regime, sex, carotenoid supplementation and resveratrol supplementation. Tank was included as random factor.

Final model	Estimate	SE	DF	*t*	*p*
Resveratrol (Y)	52.861	28.24	38	1.872	0.070
Regime (A)	-3.529	28.10	36	-0.122	0.903
Carotenoid (L)	23.350	28.43	37	0.821	0.417
Sex (M)	-3.952	30.15	48	-0.131	0.896

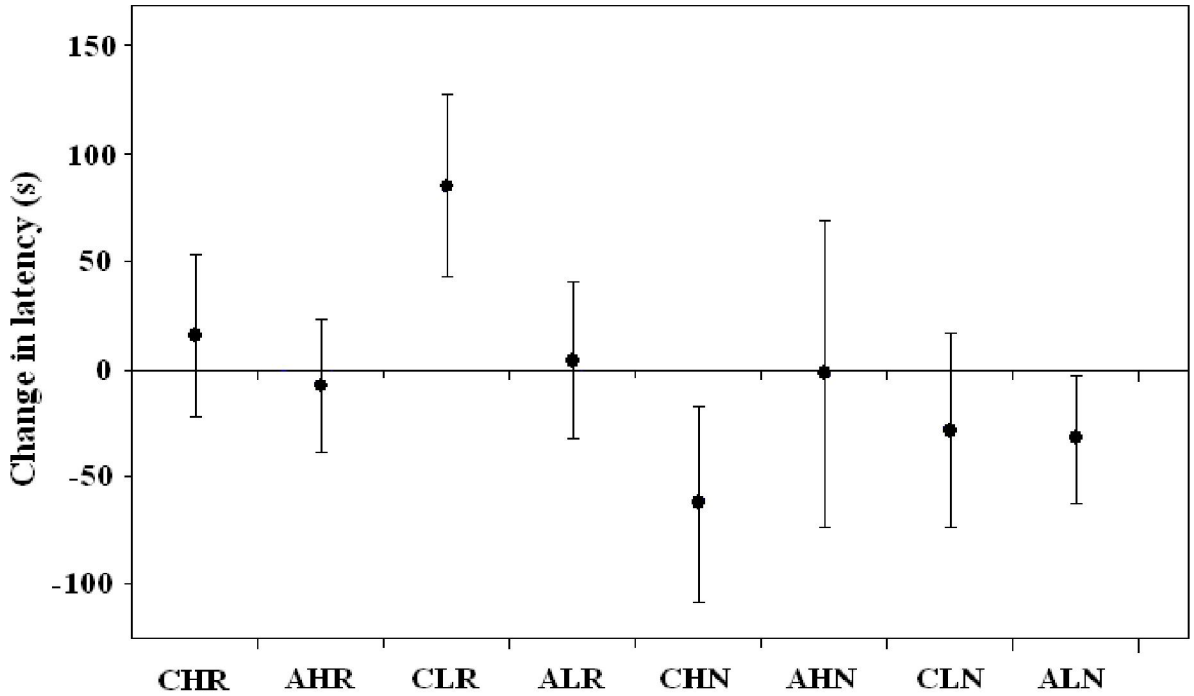


Figure 6.9 Mean ± s.e. Change in latency in seconds to leave the starting position over the experimental period for all sticklebacks in relation to the eight feeding treatment groups. High carotenoid + resveratrol compensatory growth, high carotenoid + resveratrol *ad libitum* growth, low carotenoid + resveratrol compensatory growth, low carotenoid + resveratrol *ad libitum* growth, high carotenoid no resveratrol compensatory growth, high carotenoid no resveratrol *ad libitum* growth, low carotenoid no resveratrol compensatory growth and low carotenoid no resveratrol *ad libitum* growth denoted CHR, AHR, CLR, ALR, CHN, AHN, CLN and ALN respectively.

6.5 DISCUSSION

6.5.1 Compensatory growth response

In accordance with previous work on sticklebacks (Álvarez & Metcalfe 2005; Inness & Metcalfe 2008) and the results of the previous experiments (Chapters 2 and 3), the present study found that dietary restriction in early life resulted in a compensatory growth response after the food restriction was uplifted. A four-week period of food restriction affected the growth trajectory of the fish during the food restriction period itself but also subsequently up until four weeks after this food restriction had been uplifted. This deviance in growth trajectories during the food restriction was buffered by the subsequent compensation in terms of both length and mass. Therefore, the compensatory growth regime fish compensated in terms of both energy reserves and skeletal growth.

6.5.2 Exploratory activity

Females that had exhibited compensatory growth incurred a significant reduction in their exploratory drive with age, which was measured in terms of the number of zones entered in the open field tank. However, this effect was sex specific as there was no reduction in the number of zones explored with age in the males. This result suggests that perhaps the physiological costs of reproductive maturation are different between males and females. The females may have been required to allocate more resources into reproduction than the males. If so, this may explain why this trade-off between growth and somatic maintenance was absent in the males. An alternate reason for this sex-specific trade-off may be associated with their breeding behaviour. As the males had become sexually mature by the time of the second trials (they were expressing full breeding colouration), perhaps the males had a greater motivation to search for breeding sites whereas the females may have become less active as they matured and became gravid.

However, it is also possible that the general lack of reduction in exploratory activity across the 19-week experimental period in all treatment groups suggests that the use of exploratory activity as an age-related cognitive marker in sticklebacks is inappropriate. Although exploratory activity has been successfully used as an age-related marker in rodent studies; the use of the open field paradigm to assess age-related reductions in exploratory drive in fish is restricted to only one study in *Nothobranchius furzeri*

(Valenzano et al. 2006b). Other fish studies that have successfully used exploratory activity in an open field paradigm have related this to other factors such as exposure to predators or light-dark regimes, as opposed to being used specifically as an age-related marker (Archard & Braithewaite 2010; Rosemberg et al. 2011).

6.5.3 Anxiety-related behaviour

Thigmotaxis is a well-validated index of anxiety in animals, most commonly used in rodent models but becoming more popular in fish studies (Simon et al. 1994; Ahmad & Richardson 2013). Resveratrol supplementation only reduced thigmotaxis in the fish that had not been subjected to the compensatory growth regime, which suggests that resveratrol reduced anxiety in these *ad libitum*-fed sticklebacks. Although fish fed resveratrol on the compensatory growth regime maintained the same degree of thigmotaxis across the 19-week experimental period, the fish that had not been fed resveratrol incurred a reduction in thigmotaxis. These results produce conflicting perspectives on the abilities of resveratrol in reducing anxiety-related behaviour. Immediate treatment with resveratrol after a blunt head trauma has been previously postulated to have beneficial effects on trauma-induced anxiety behaviour in Wistar albino rats (Sönmez et al. 2007). However, a limitation of that study was that anxiety was inadequately inferred as solely a reduction in exploratory activity (Sönmez et al. 2007). Regardless, resveratrol has also had positive effects on anxiety-related behaviour in prenatally-stressed albino Wistar rat offspring subjected to an open field test on their 21st postnatal day (Sahu et al. 2013). Administration of resveratrol significantly increased the amount of time spent in central squares as opposed to the peripheral squares of an open field arena which inferred a reduction in thigmotaxis and therefore implies a reduction in anxiety (Sahu et al. 2013). However, contrasting findings in the literature with regard to resveratrol's ability to reduce anxiety-related behaviour could be the result of species-specific effects alongside possible differences in the administration of resveratrol (e.g. chronic versus acute supplementation, which may modulate resveratrol's efficiency as an anti-ageing agent). For instance, it is possible that the increased dose of resveratrol (3000μg g^{-1}) administered in period 1 of the present study (which was calibrated to account for the reduced amount of feed received by the compensatory growth regime fish) may have caused resveratrol to exert pro-oxidant activities. Indeed, resveratrol's abilities as a pro-oxidative agent have been previously demonstrated under certain circumstances (Win et al. 2002; Ahmad et al. 2005; Alarcónde la Lastra & Villegas 2007). This highlights the importance of obtaining effective and safe

doses of resveratrol with regard to its reputation as a neuroprotectant and its clinical implications for human use. However, one recent safety study conducted on resveratrol in laboratory rats found no negative toxicity effects of a high dose of resveratrol (Williams et al. 2009). Nevertheless, although high doses of resveratrol are well tolerated and non toxic in laboratory rats (Williams et al. 2009), this finding cannot be generalised across taxa.

There were no differences between feeding treatments in the latency of the fish to leave the starting position in the open field tank. This is perhaps unsurprising as invariance in immobile behaviour during the assessment of novel environments has also been found across individuals in numerous studies using zebrafish, mice, rats and voles *Microtus socialis guentheri* (Drai et al. 2001; Eilam et al. 2003; Kalueff et al. 2006; Stewart et al. 2012). This suggests that stopping and immobile behaviour is a consistent feature of exploratory behaviour across species which may not be solely anxiety-evoked but may also be indicative of behaviour associated with decision-making and information-processing (Stewart et al. 2012). Therefore, these potentially contrasting and multiple reasons for immobile behaviour may explain the lack of differences found in the present study between feeding treatments with regard to the latency of the fish to leave the starting position.

6.5.4 Additional remarks

Carotenoids did not have a significant effect on any of the four markers measured to infer rate of cognitive ageing in the present study. This is in accordance with a longitudinal study where older woman consuming a carotenoid-rich diet for a 10 year period showed no benefits of this in terms of their cognitive performance with age (Kang & Grodstein 2008). Although there has been a number of other studies in humans that have found positive effects of long-term intake of carotenoid-rich food on cognition, there are important factors that press judgement on their findings and conclusions (Devore et al. 2013). For instance, previous studies tend to rely on their participants to self report details of their diet which may increase error in dietary information. Secondly, long-term studies investigating cognitive function may be confounded by alternative factors that persist over time such as a participant's educational history throughout the course of the study (Grodstein et al. 2003).

CHAPTER 7 - GENERAL DISCUSSION

7.1 Compensatory growth and its effects on subsequent performance and oxidative stress

Throughout a series of experimental investigations using three-spined sticklebacks in chapters 2-6, this thesis has demonstrated that early food availability can alter juvenile growth trajectories. Individuals that had been subjected to a restricted dose of food in early life exhibited a compensatory growth response once food availability had been restored (Chapters 2, 3 and 6). This increase in growth rate allowed these juveniles to reach a comparable body size to controls by the onset of sexual maturity. However, previous research indicates that this accelerated growth rate can result in a range of long-term costs (Criscuolo et al. 2011; Dmitriew 2011; Lee et al. 2012; Lee et al. 2013). Overall, the results of the experiments in chapters 2-6 show that compensatory growth had a negative effect on some, but not all aspects of subsequent performance in adulthood. Alongside oxidative stress (Chapters 2 & 3), numerous life history traits were measured, including female reproductive investment (Chapter 5), male sexual signal investment (Chapter 4) and senescence by measuring a number of age-related markers in adulthood (Chapter 2 & 6).

Firstly, compensatory growth led to marginally reduced cognitive performance in an active avoidance task (Chapter 2). This finding supports the work of Fisher et al. (2006), which demonstrated that growth compensation resulted in long-term negative consequences for cognitive function in adult zebra finches. Oxidative stress may be an underlying physiological mechanism responsible for such a growth-performance trade-off (Fisher et al. 2006). For instance, Kim et al. (2011) showed that a higher chick mass growth rate, from hatching to day 8, resulted in reduced blood resistance to oxidative attack in yellow-legged gulls *Larus michahellis*. These results accompany the growing evidence that demonstrates that rapid growth can influence oxidative stress status in zebra finches, wild king penguins and rainbow trout *Oncorhynchus mykiss* (Alonso-Alvarez et al. 2007; Geiger et al. 2011; Almaida-Pagán et al. 2012). Accelerated growth rates can result in faster cellular degradation and oxidative damage (Nussey et al. 2009; Lucas-Sánchez et al. 2011; Guerra et al. 2012). The brain is comprised largely of polyunsaturated fatty acids which are highly susceptible to free radical attack during periods of high oxidative stress, such as during rapid growth (Ulmann et al. 2001; Geiger et al. 2011; Almroth et al. 2012). Indeed, compensatory growth was found to influence some aspects of oxidative stress status in chapters 2 and 3. For example, while growth regime had no effect on oxidative

damage to proteins, it did result in differences in the upregulation of two important endogenous antioxidants, GPx and SOD (Chapter 3). However, these differences were complex in that they were further influenced by the amount of exogenous antioxidants supplemented in their diets. For example, fish fed a diet low in carotenoids and on a compensatory growth regime upregulated GPx and SOD significantly more than compensatory growth regime fish that had received a high carotenoid diet (Chapter 3). In circumstances when resources are limiting, an increased upregulation of SOD and GPx would have come at a cost to investment elsewhere, such as the maintenance of the neurophysiological processes underlying cognition. As a whole, these findings support the hypothesis that the evolution of growth trajectories may be constrained by oxidative stress (Kim et al. 2011). In the case of the results of chapter 2, as it is crucial to healthy cognitive functioning that redox balance is maintained, accelerated growth may have resulted in a trade-off which affected subsequent cognitive performance due to higher levels of oxidative stress (Glade 2010).

Additionally, in accordance with previous work using the same population of three-spined sticklebacks (Álvarez & Metcalfe 2005; Lee et al. 2010), compensatory growth was also found to negatively influence swimming performance in chapter 2. This finding also supports the work of Farrell et al. (1997) that demonstrated a marked trade-off between growth rate and swimming performance in growth-enhanced coho salmon *Oncorhynchus kisutch*. In addition, Lee et al. (2010) proposed that compensatory growth may negatively affect muscle structure leading to reduced locomotor ability as accelerated growth rates have been shown to restrict healthy muscle development (Christiansen et al. 1992). For this reason, alongside the aforementioned discussions highlighting the links between accelerated growth and oxidative stress, these points may have contributed to the reduced swimming performance by the sticklebacks fed under a compensatory growth regime in chapter 2.

It has been well documented that there can be reproductive fitness benefits of reaching a size threshold in time for the breeding season (Dickerson et al. 2002; Fokidis et al. 2007). However, more recently, it has also come to light that reaching a large body size through compensatory growth can also be associated with costs to reproduction (Auer et al. 2010a; Lee et al. 2012). Despite this, compensatory growth in the present study was not found to incur any obvious reproductive fitness consequences in the male three-spined sticklebacks in terms of investment in sexual ornamentation (Chapter 4), nest building ability (Chapter

4) or male attractiveness in a female mate choice experiment (Chapter 5). This may be the result of the experimental animals being tested under laboratory conditions so that they did not bear the same potential costs of compensatory growth that would be expected in the wild such as avoiding predation (Hector & Nakagawa 2012). The absence of factors such as these may explain the absence of the importance that a compensatory growth regime had on influencing reproductive investment in chapter 4. Although one recent study in three-spined sticklebacks did find significant costs to reproductive investment following compensatory growth (Lee et al. 2012), these were achieved when photoperiod manipulations were used in order to reduce the perceived time available to complete this growth compensation before the onset of the breeding season (Lee et al. 2012). Therefore, perhaps such strong effects were not achieved in chapter 4 due to the sticklebacks having a larger time period available in order to achieve and recover from full growth compensation prior to commencing the breeding season.

Early growth conditions have been shown to determine a mother's allocation of her total energy resources into the number and size of her offspring (Taborsky 2006a; Taborsky 2006b). In the present thesis, it was initially expected that compensatory growth may negatively impact female reproductive output due to a greater allocation of limited resources invested into accelerated growth rather than into reproduction. However, this was not the case; compensatory growth did not reduce female reproductive output, measured in terms of clutch mass (Chapter 3). Juvenile growth conditions have been shown to influence key reproductive traits such as reproductive rate and offspring size in female mouthbrooding cichlids *Simochromis pleurospilus* (Taborsky 2006b). However, most interestingly, the female mouthbrooding cichlids that had been raised with food limitations in early life were found to produce larger young than females that had been raised with more favourable food conditions (Taborsky 2006a). It was proposed that maternal investment was adjusted so that the mothers prepared their young for similar environmental conditions as they had encountered themselves as juveniles (Taborsky 2006a). Perhaps this was the case in the female three-spined sticklebacks in chapter 3, whereby the mothers may have compensated for their poor earlier food availability by maximising their investment in their clutch to match that of mothers that had experienced a plentiful supply of food during early development. However, more recent evidence affirms that compensatory growth can also reduce reproductive investment. For example, compensatory growth resulted in a 20% decline in offspring production in Trinidadian guppies (Auer et al. 2010a) and led to a reduced clutch size in three-spined sticklebacks

(Lee et al. 2012). The lack of effect that compensatory growth had on reproductive investment in chapter 3 is somewhat surprising given that compensatory growth is associated with increased oxidative stress (Geiger et al. 2011) and female reproductive physiology is heavily reliant on oxidative balance (Rizzo et al. 2012).

Nevertheless, in accordance with Auer et al. (2010a) and Lee et al.(2012), compensatory growth did influence one aspect of the females' reproductive behaviour, which was the time the females spent actively choosing between males (Chapter 5). There was a stronger difference in "choosiness" between compensatory growth and *ad libitum* growth regime females in the low carotenoid group (Chapter 5). Notably, it has been suggested that latency to choose a mate is affected by numerous search costs and therefore females will adjust their mating behaviour according to their own condition (Jennions & Petrie 1997; Härdling & Kokko 2005). Although there is a growing number of state-dependent mate choice models that predict reduced choosiness in low-quality females, there is still little empirical evidence to support this prediction (Fawcett & Johnstone 2003; Härdling & Kokko 2005). However, more recently this hypothesis has been supported in zebra finches, where reduced female quality was found to alter choosiness (Holveck & Riebel 2010). Therefore, it is plausible that although the compensatory growth females in chapter 5 were able to maintain reproductive output similar to that of *ad libitum* growth females, they may have been in poorer condition and perhaps this came at a cost during mate choice behaviour and elucidates why they chose males less discriminately.

Finally, compensatory growth led to reduced exploratory behaviour and increased thigmotaxis in an open-field task (Chapter 6). These findings support the work of Krause & Naguib (2011), which demonstrated that compensatory growth affected adult exploratory behaviour in zebra finches. Individual finches that had undergone greater compensatory growth were less active during a spatial test and also exhibited reduced exploratory activity (Krause & Naguib 2011). Exploratory activity has been closely linked with foraging behaviour (Herborn et al. 2010) and therefore the reduced exploratory activity shown in the females that had undergone compensatory growth in chapter 6 suggests that this may have influenced survival and fitness if the experiment had been undertaken in a more natural context.

Overall, as the results have shown that compensatory growth had little impact on reproduction in both males and females in this thesis (Chapters 3, 4 & 5), perhaps the

advantages of obtaining a comparable body size to *ad libitum* growth peers in time for the breeding season outweighed the importance of the physiological costs and reduced performance (Chapters 2, 3 and 6) associated with undergoing this compensatory growth strategy.

7.2 Dietary resveratrol and carotenoid supplementation and its effects on subsequent performance and oxidative stress

The diets of the individuals in the experiments discussed above (Chapters 2-6) were manipulated further to investigate whether the availability of two dietary antioxidant supplements, resveratrol and carotenoids, were able to alleviate any negative affects associated with compensatory growth and improve subsequent performance and oxidative stress status. I will now discuss their influence on each of the measured traits.

7.2.1 Growth

There was no evidence to suggest that resveratrol and carotenoids, either fed independently or combined in the diet, were able to further modify the three-spined sticklebacks' growth trajectories (Chapters 2, 3 and 6). This is in accordance with recent results in yellow-legged gulls, where the supplementation of an alternative dietary antioxidant, vitamin E, was not found to affect chick growth (Noguera et al. 2010). Moreover, this result has been confirmed more recently again in yellow-legged gulls, where the supplementation of vitamins C and E, had no effect on growth, in terms of average body mass (Kim et al. 2013). Additionally, the supplementation of the carotenoid, astaxanthin, for an 84-day period did not influence growth rate in rainbow trout (Rehulka 2000). However, the above findings are contradictory to the work of Orledge et al. (2012) that found that birds supplemented from early life with a combination of carotenoids and vitamin E grew faster and were heavier than controls. It was postulated that these positive effects on growth were synergistic as it was in combination with one another that these supplements were able to maximise growth rates and allow the individuals to reach a larger size (Orledge et al. 2012).

Previous studies investigating the independent effects of dietary carotenoid supplementation on growth have produced inconsistent results to date (Chien & Shiau 2005). For example, although carotenoid supplementation in the diet of black tiger shrimps

Penaeus monodon has been shown to yield positive effects on growth (Niu et al. 2012), it has also been shown to lack any significant effects (Boonyaratpalin et al. 2001). Research investigating the effects of carotenoids on growth in aquatic species has been predominantly motivated by the aquaculture industry seeking to maximise growth rates and production, with a distinct lack of focus on the evolutionary relevance of dietary supplements influencing traits such as growth. This is an important gap in the literature that needs to be filled.

It has been postulated that growth may be more greatly affected by the overall antioxidant status of an individual rather than restricted to the antioxidant status of single tissues (Catoni et al. 2008). This has led to the suggestion that polyphenolic antioxidants may play an important role in growth as they have the highest antioxidant potency across tissues (Catoni et al. 2008). However, dietary-ingested resveratrol did not influence growth rate in the present study (Chapters 2, 3 & 6). This result is in line with recent findings in male Sprague-Dawley rats, where resveratrol played no role in influencing body weight, following a similar compensatory growth feeding protocol to the one adopted in this thesis (Zheng et al. 2012).

In the present thesis, the lack of effect of the dietary supplementations on growth suggests that the three-spined sticklebacks did not preferentially allocate these supplementary antioxidants directly into achieving a larger body size. As three-spined sticklebacks are a sexually dimorphic species, one explanation for the lack of effect these supplements had on growth may be that the males alternatively invested their surplus carotenoids into elaborating their sexual ornamentation, which is an important determinant of success in mating (Milinski & Bakker 1990). Additionally, perhaps the females invested their surplus carotenoids into their egg production, as carotenoids are known to have beneficial effects during embryonic development in fish (Luzbens et al. 2003). The results of chapter 4 support this hypothesis, which investigated to what extent the supplementation of resveratrol and carotenoids in the diet affected the strength of the males' sexual signal. Males that had been provided with a high carotenoid diet from early life displayed brighter nuptial throat colourations throughout the breeding season (Chapter 4). However, although resveratrol had no effect on regulating investment in nuptial throat colouration (Chapter 4), it was found to influence other aspects such as cognitive performance (Chapter 2).

7.2.2 Cognitive and swimming performance

In chapter 2, compensatory growth was found to negatively affect two age-related markers, cognitive and swimming performance. Chapter 2 investigated to what extent these negative effects were alleviated by the supplementation of resveratrol and carotenoids in the diet.
Firstly, both resveratrol and carotenoid supplementation from early life did not influence subsequent swimming performance in adulthood (Chapter 2). However, this is not the first study that has not found improved locomotory performance with a diet enhanced in dietary antioxidants. For example, no differences in fight performance were found in adult budgerigars that received a diet either rich or poor with antioxidants (Larcombe et al. 2008). Larcombe et al. (2008), suggested that the poor antioxidant diet budgerigars had maximised their efforts in their flight performance at the expense of their oxidative stress status post-exercise. Indeed, this exertion did result in an increased oxidative stress status in the poor antioxidant diet budgerigars in comparison with those on the rich antioxidant diet (Larcombe et al. 2008). These findings are consistent with the swimming performance results presented here in chapter 2. Although the fish fed a diet lacking in resveratrol had a comparable swimming performance to those individuals that had received resveratrol, this consequently led to differences in post-exercise oxidative stress status between the two groups. The fish that had not received resveratrol had increased their levels of the endogenous antioxidant enzyme SOD, perhaps in order to cope with this high-energy performance during the swimming trials. Nevertheless, these results are in contrast to the conclusions of Pike et al. (2010b), who found that a low dietary carotenoid intake negatively impacted swimming performance in the same population of three-spined sticklebacks that were used in this thesis. Additionally, in male budgerigars, locomotor capacity was found to be significantly greater in males supplemented with a diet high in antioxidants that included carotenoids (Arnold et al. 2010). However, in line with the work of Arnold et al. (2010), a diet high in carotenoids resulted in a significantly faster escape response from a warning stimulus during the active avoidance task (Chapter 2). Arnold et al. (2010) suggested that the mechanism by which the antioxidant supplemented diet improved escape flight performance in the male budgerigars was through the abilities of the dietary-derived antioxidants to combat the negative effects associated with oxidative stress and therefore protect the tissues associated with the escape response in the budgerigars (Arnold et al. 2010). This may indeed be one of the factors resulting in the faster escape response in the high carotenoid fed sticklebacks during the active avoidance task in chapter 2.

Secondly, resveratrol played a beneficial role in influencing correct response rate during the active avoidance task, which was used to assess cognitive performance in adulthood (Chapter 2). This result reinforces a growing number of studies that provide evidence that resveratrol maintains and, in some circumstances, even improves cognitive functioning with age (Valenzano et al. 2006b; Yu & Li 2012; Liu et al. 2012). It is an interesting finding that resveratrol mitigated the negative effects that compensatory growth played on cognitive performance, but not on aspects related to exercise performance in terms of swimming endurance and escape speed (Chapter 2). These results suggest that resveratrol protects neurons and associated cells more efficiently than structures related to exercise such as muscles. To understand results like this better, it would be important to expand the knowledge of resveratrol's biodistribution in organs and tissues *in vivo*. Indeed, there has been research to suggest that the beneficial effects of resveratrol are highly restricted by its poor bioavailability *in vivo* (Vang et al. 2011; Amri et al. 2012). However, in conflict with this hypothesis, Zheng et al. (2012) demonstrated that resveratrol protected skeletal muscle from the negative effects of catch-up growth in male Sprague-Dawley rats. Resveratrol was found to upregulate the activity levels of endogenous antioxidant enzymes that were able to reduce levels of ROS in muscle tissue (Zheng et al. 2012). This subsequently resulted in improved mitochondrial respiration which prevented systemic insulin resistance (Zheng et al. 2012), a detrimental side-effect of catch up growth in these rats (Chen et al. 2011). Existing evidence that further links resveratrol supplementation to improvements associated with either muscle structure or function is limited (Dirks-Naylor 2009). Most of the limited evidence available is restricted to emphasising resveratrol's effects on the cardiovascular system in particular (Li et al. 2012). Therefore, this area of research should be further explored.

7.2.3 Male and female oxidative stress status and female egg investment

Early ecological research into the positive effects associated with an increase in carotenoid intake was focused mainly on its influence on sexual ornamentation and its effect on reproductive success (Lozano 1994; von Schantz et al. 1999) . More recently there has been a growing interest outside the area of ornamental carotenoids and the focus has shifted towards the potential role of carotenoids as antioxidants (Svensson & Wong 2011; Simons et al. 2012). However, the existence of any antioxidant effects of carotenoids is still debated in the literature (Hartley & Kennedy 2004; Costantini & Møller 2008). Chapter 3 investigated whether resveratrol and carotenoid supplementation reduced

oxidative stress in both male and female three-spined sticklebacks. In addition, the chapter addressed whether resveratrol and carotenoid supplementation did indeed reduce oxidative stress in females and whether this was reflected in a) her egg clutch investment and b) her egg quality in terms of the antioxidant capacity of her unfertilised eggs. In the past, the interpretations of many oxidative stress results have been restricted by the limited components of oxidative stress that were measured (e.g. Alonso-Alvarez et al. (2004); Wiersma et al. (2004)). Therefore, the importance of appropriately measuring oxidative stress has been increasingly recognised (Costantini & Verhulst 2010; Selman et al. 2012). The laboratory experiments in chapters 2 and 3 used three assays to assess oxidative stress effectively. These included two measures of endogenous antioxidant enzyme activity (SOD and GPx) that are associated with oxidative protection, alongside a measure of oxidative damage to proteins (protein carbonyl content).

The level of oxidative damage that the three-spined sticklebacks incurred was influenced by their relative intake of carotenoids, where a diet higher in carotenoids significantly reduced oxidative damage to proteins (Chapter 3). This result suggests that although carotenoids appear to be unimportant antioxidants for birds (Costantini & Møller 2008; Perez-Rodriguez 2009), these findings should not be generalised across all taxa. Additionally, the differences in endogenous antioxidant enzyme upregulation of SOD and GPx in chapter 3 were complex, with interactions emerging separately between growth regime and the two different dietary supplements. However, a key conclusion obtained from these findings was that dietary antioxidants such as resveratrol that are not present naturally in the diet of three-spined sticklebacks might in fact promote the production of ROS (Chapter 3). For example, although resveratrol has been recently shown to increase oxidative stress resistance in *C. elegans* strains, the antioxidative abilities of resveratrol were capped at 50μM and higher concentrations failed to improve resistance (Chen et al. 2013). These positive antioxidant effects of resveratrol at this lower concentration support the hypothesis that resveratrol can exert pro-oxidant effects at higher concentrations (Chen et al. 2013).

It is well known that particular antioxidants can have strong synergistic interactions between one another but combinations of certain antioxidants can also be counterproductive and can even produce negative effects on life history traits (Snell et al. 2012). Possibly, the lack of an additive effect of a diet containing a combination of resveratrol and carotenoids throughout chapters 2-6 is the result of a negative interaction

between the supplements. Further to this, perhaps differences in the biochemistry of resveratrol and the two carotenoids, lutein and astaxanthin, may provide an explanation for the division of benefits these supplements often had in this thesis towards different traits. There may have been asynchrony in the bioaccumulation of these supplements which may explain why there were no additive effects of resveratrol and carotenoids on any one trait when they were fed in combination in the diet of the sticklebacks. This highlights the importance of examining the biochemical effects of ingesting various combinations of specific compounds from different antioxidant classes at any one time, such as the combined supplementation of resveratrol and carotenoids in the experiments in this thesis. For instance, in combination they may have enhanced the absorption efficiency of one another or in contrast they may have outcompeted one another for absorption (Reboul et al. 2007). For example, it has been demonstrated that lutein absorption is impaired by up to 30% in human intestinal cells when ingested in a meal containing a mixture enhanced in polyphenols and other carotenoid species (Reboul et al. 2007). In contrast, vitamin E and C supplementation did not influence lutein absorption (Reboul et al. 2007). These findings illustrate the significance of understanding interactions among antioxidants when attempting to produce diets that are hypothesised to optimally enhance life history traits under stressful conditions.

One such life history trait that was hypothesised to be positively affected by dietary antioxidant availability in this thesis was reproduction. Indeed, maternal dietary antioxidant availability of carotenoids did affect female reproductive effort (Chapter 3). Females fed a high carotenoid diet lacking in resveratrol produced significantly larger clutch masses than females that received resveratrol only, or those that had been fed a low carotenoid diet (Chapter 3). These results suggest that carotenoids were a key modulator in female egg investment. Resveratrol did not play an additive role alongside the carotenoids in benefiting female reproductive output (Chapter 3). There are mixed results of the effects resveratrol has on reproductive physiology (Kyselova et al. 2003; Collodel et al. 2011). However, to date, there is little evidence to suggest that resveratrol improves reproductive fitness (Henry & Witt 2006). Nevertheless, young mice fed resveratrol for 12 months retained the capacity to reproduce whilst age-matched controls produced no pups (Liu et al. 2013). Although in contrast, resveratrol supplementation had no effects on female reproduction in tephritid fruit flies *Anastrepha ludens* (Zou et al. 2009). The dietary supplementation of carotenoids and resveratrol had no influence on the antioxidant capacity of either the female sticklebacks or the egg clutches (Chapter 3). However, these

findings are surprising as carotenoids are often regarded as important antioxidants in eggs (Blount et al. 2002a; Blount et al. 2002b). Nevertheless, they support the work of Costantini (2010), who found that different levels of antioxidants supplemented in the diet of breeding female pigeons *Columba livia* had no effect on the antioxidant capacity of either the yolk or albumen components of their eggs.

Resveratrol played a role in mediating reproductive behaviour in chapter 5, whereby females supplemented with resveratrol spent significantly more time associating with males than females that had not been fed resveratrol. The process of mate choice has been suggested to be costly in many species, since the assessment and comparison of potential mates is an energetic process which is likely to increase oxidative stress resulting from this increased physical activity (Pomianowski 1987; Byers et al. 2005; Toomey & McGraw 2012). Therefore, resveratrol may have facilitated active choice in females through beneficial effects associated with its antioxidant properties (Fremont 2000; Gulcin 2010; Cai et al. 2011).

7.2.4 Male reproductive investment

Chapter 4 examined the effect of dietary antioxidant availability of resveratrol and carotenoids on resource allocation and reproductive decisions in the male three-spined sticklebacks during their first breeding season. Sexual signal investment was measured alongside three combined aspects of male nest building ability. Males fed a diet high in carotenoids displayed brighter sexual signals at two crucial stages during the breeding season in comparison with low carotenoid fed males (Chapter 4). This result is in line with numerous other studies that show that dietary carotenoid supplementation positively affects the expression of carotenoid-dependent sexual ornaments (Smith et al. 2007). More interestingly, it has been suggested that the expression of ornaments may depend on the oxidative stress status of the individuals (von Schantz et al. 1999). In support of this, the results showed that the redder males had lower oxidative damage to proteins at the end of the breeding season (Chapter 4). This result provides evidence that carotenoid-dependent ornamentation is linked with oxidative stress status. Therefore, the three-spined stickleback's nuptial throat colour intensity was a true indicator of male condition in chapter 4. In comparison, carotenoid supplementation did not enhance plumage colouration or reduce oxidative damage in nestling blue tits *Cyanistes caeruleus* (Larcombe et al. 2010b). However, as carotenoids in that study did not influence plasma MDA, a by-

product of lipid peroxidation (Larcombe et al. 2010b), it lends support to the idea that carotenoids are not as important antioxidants in birds as they seem to be in the case of three-spined sticklebacks. Chapter 4 provided no evidence that non-pigmentary antioxidants (in this case resveratrol) protect carotenoid pigments from oxidative decolouration, increasing their availability for their deposition in sexual ornaments, as proposed by Hartley & Kennedy (2004). In line with this result, other non-pigmentary antioxidants that have also not been found to provide antioxidant protection of carotenoid colourations are vitamin E and α-tocopherol (Karu et al. 2008; Larcombe et al. 2010b).

The males that received a diet high in carotenoids took significantly less time to both begin and complete nest building (Chapter 4). This supports the work of Pike et al. (2007c) that also found that males with a greater access to carotenoids performed better in energetically expensive parental care tasks. In chapter 4, faster nest building males had brighter throats, lower oxidative damage to proteins, and also utilised a larger number of threads to construct their nests. These results promote the idea that oxidative stress provides a physiological explanation for the link often found between carotenoids and investment in male reproduction (Locatello et al. 2006; Pike et al. 2007c; Pike et al. 2010a). Resveratrol played no role in influencing male reproductive investment (Chapter 4). However, in hindsight it would have been interesting to have investigated whether resveratrol improved male fertility by allowing the males to fertilise the female eggs. There is minimal evidence to suggest that resveratrol can have beneficial effects on reproductive behaviour, but there is some evidence to suggest that it can benefit reproductive physiology, particularly at the cellular level. For instance, resveratrol has been found to increase sperm motility and protect human sperm and rat germinal cells from oxidative damage (Collodel et al. 2011). However, it is unclear whether this would result in greater reproductive success, since male rats exposed to resveratrol from nursing have been shown to exhibit decreased plasma testosterone concentrations and reduced sociosexual behaviour (Henry & Witt 2006).

7.2.5 Male sexual attractiveness

Chapter 5 investigated whether a compensatory growth regime reduced a male's sexual attractiveness and also whether the supplementation of resveratrol improved male attractiveness in terms of female mating preference. The supplementation of resveratrol did not increase the expression of the red nuptial colouration (Chapter 4), and it is therefore perhaps unsurprising that resveratrol did not influence female mate preference during the

mate choice trials (Chapter 5). This finding is in accordance with the work of Blount et al. (2003a), which found that female zebra finches did not prefer males reared on a diet higher in both vitamins A and C. In both studies the males did not differ in their external appearance to the females in terms of the brightness of their sexual ornaments (red throats in the case of sticklebacks, and red bills in the case of the zebra finches) (Blount et al. 2003a). These results suggest that the females did not receive any alternative (and potentially important) mate cues mediated by resveratrol (chapter 5) and vitamins A and C (Blount et al. 2003a) that were independent of the carotenoid-based signal. Perhaps resveratrol would increase sexual attractiveness in other species where ornament colouration is not a predominant cue in mate choice.

7.2.6 Exploratory behaviour

Chapter 6 investigated whether the supplementation of resveratrol and carotenoids reduced the rate of ageing in terms of exploratory and anxiety-related behaviour in an open field test, measured during early life and again in adulthood. Males that were not fed resveratrol had the greatest increase in exploratory activity across the experimental period in terms of total number of zones explored and zone borders crossed (Chapter 6). This was in contrast to expectations, as it was hypothesised that the resveratrol treatment fish would exhibit the greatest increase in exploratory activity with age. The basis of this hypothesis was due to growing evidence in the literature demonstrating that resveratrol had neuroprotective properties (Richard et al. 2011). However, the opposite results that were produced in chapter 6 may be due to the increased dose of resveratrol (3200µg g^{-1}) in the supplement given to the three-spined sticklebacks during period 1, which may have caused resveratrol to exert pro-oxidant activities. Therefore, the resveratrol treatment fish may have potentially been in poorer condition due to higher levels of oxidative stress and this may have influenced their exploratory drive, although this remains speculation since the oxidative stress status of these fish was not assessed. Alternatively, as exploratory drive is associated with foraging, it may be that the fish not fed resveratrol had increased exploratory behaviour as they were seeking food to cope with higher nutritional requirements. In support of this, adult female zebra finches raised under different nutritional conditions from early life have also been shown to differ in their exploratory behaviour in adulthood (Krause et al. 2009). The females raised under a poorer quality diet showed increased exploratory activity in terms of the latency to leave their starting box in the trials (Krause et al. 2009). However, as a hidden food source was present during these

trials, it was suggested that these poor quality females may have been exhibiting this more risk taking behaviour as a foraging strategy to compensate for their poor quality diet in early life (Krause et al. 2009).

In terms of thigmotaxis, resveratrol supplementation did not improve this anxiety-related behaviour with age (Chapter 6). In fact, conflicting results were produced that were dependent on which growth regime the fish experienced (Chapter 6). In comparison, anxiety-related behaviour in male Long Evans and Wistar rats was unaffected by the administration of a series of injections containing 3mg/kg doses of resveratrol (Patisual et al. 2009). Resveratrol did not influence the number of arm entries in an elevated plus maze test or the number of entries into a light chamber during a light/dark box test (Patisual et al. 2009). However, resveratrol administration in prenatally stressed Wistar rats increased the number of central square entries undertaken in an open-field test (Sahu et al. 2013). Further reductions in thigmotaxis were demonstrated in the resveratrol treatment group, whereby these rats also took significantly less time to reach a target quadrant (Sahu et al. 2013). It was postulated that these displayed reductions in anxiety-related behaviour were related to resveratrol's potent antioxidant abilities in the hippocampal regions of the brain (Sahu et al. 2013). However, oxidative stress was not measured in the rats, and these speculations were based on the antioxidant abilities of resveratrol demonstrated in other studies (Mokni et al. 2007; Ates et al. 2007). Notably, as Sahu et al. (2013) provided their rats with a higher dose of resveratrol (10 mg/kg) in comparison with the lower 3mg/kg dose used in Patisual et al. (2009), these differences in dose concentrations may have produced the differences found in their anxiety-related behavioural results.

7.3 Limitations and conclusions

A recent review by Selman et al. (2012) emphasised the shortcomings of researching the role that oxidative stress plays in shaping life histories in laboratory animals as opposed to using field studies on wild animals. One such concern is that laboratory studies investigating life history trade-offs are often assessed under unnatural conditions whereby individuals are provided with a bountiful food supply alongside a safe environment free from predators. However, this thesis has partially overcome this problem by subjecting the three-spined sticklebacks to a restricted supply of food in early life so that once food availability was restored these fish would have faced resource allocation challenges similar to those they may have expected to face in the wild. This has been particularly useful in

developing our understanding of how resveratrol mediates life history traits, since previous experiments to date have been conducted on animals with *ad libitum* access to food (Valenzano et al. 2006b; Yu & Li 2012). This thesis demonstrated that resveratrol (alongside carotenoids) mediated numerous life history traits throughout a large proportion of the three-spined stickleback's lifespan. The results of this thesis have broad implications beyond ecology and life history evolution as the connection between nutrition and ageing through oxidative stress is paramount with regards to human health.

The results of this thesis have helped to extend our knowledge of how dietary antioxidants can influence life history events through mediating oxidative stress. However, future studies may wish to consider using a study species which can be sampled multiple times for oxidative stress status. In the present thesis, in order to evaluate oxidative stress the animal had to be culled. In hindsight, it would have been interesting to have been able to assess oxidative stress status across the whole lifespan of the animal at multiple time points including immediately after the animals had fully compensated in growth. Also in order to make advances in this area of research it would also be very interesting to simultaneously investigate the bioaccumulation of dietary antioxidants across different tissues. By understanding their biodistribution within the body, this may help shed light on why their beneficial effects are found to improve some but not all traits measured in this thesis.

APPENDIX I

Food preparation

Throughout all the experiments carried out in this thesis, all fish were fed frozen chironomid larvae (nutrient composition: protein; 5%, fat; 0.7% fibre; >1%, ash; 2%, moisture; 90%), which are naturally low in carotenoids and have no added artificial antioxidants (See Appendix II for the HPLC analysis which supports this statement). These chironomid larvae were then supplemented with the dietary antioxidants in question in the present study which were resveratrol and two carotenoids, lutein and astaxanthin. If artificial antioxidants had already been present, these may have confounded the effects of these added dietary antioxidants.

Equal quantities of astaxanthin, 3,3'-dihydroxy-β,β-carotene-4,4'-dione (Carophyll Pink 10%-CWS; DSM, Basel, Switzerland) and lutein, 3,3'-dihydroxy-β,ε-carotene-diol (FloraGLO LUTEIN 10% CWS/S-TG; DSM, Basel, Switzerland), which also contains approximately 1% zeaxanthin, 3,3'-dihydroxy-β,β-carotene-diol were the source of carotenoids used in this study. The certificate of analysis for both Carophyll Pink and FloraGLO confirmed that no other antioxidants were present.

Frozen chironomid cubes were thawed without the use of water at room temperature on filter paper and placed in a sieve for one hour. The thawed chironomid larvae were then aliquoted to each cube within a cube tray in three gram divisions.

The following protocol was used to make doses for 60 low (10µg of carotenoids g^{-1} chironomid larvae) and 60 high (200µg of carotenoids g^{-1} chironomid larvae) carotenoid control (C) growth enriched chironomid cubes:

To obtain the stock solution for the low carotenoid C growth diets, 9mg of Carophyll Pink and 9mg of FloraGLO were dissolved in 200µl of 100% ethanol. Next, a 5% ethanol solution in Millipore-water was added until a stock solution of 15ml was reached. To obtain the stock solution for the high carotenoid C growth diets, 180.5mg of Carophyll Pink and 180.5mg of FloraGLO were dissolved instead.

The following protocol was used to make 60 doses of resveratrol-supplemented stock solution (415µg of resveratrol g^{-1} chironomid), which would be added to 30 low and 30 high carotenoid control growth enriched chironomid cubes accordingly:

Resveratrol (75mg), 3,4′,5 -trihydroxy-*trans*-stilbene (Novanat Bioresources Co., Ltd.) was dissolved in 200µl of 100% ethanol. Then a 5% ethanol solution in Millipore-water was added until a stock solution of 15ml was reached.

To make the correct doses for 12 low carotenoid (50µg of carotenoids g^{-1} chironomid larvae) and 12 high carotenoid (1000µg of carotenoids g^{-1} chironomid larvae) restricted growth enriched chironomid cubes, the C growth protocols were repeated with the same quantities as described above. However, instead the stock solutions were made up with a 5% ethanol solution in Millipore-water until 3ml was reached instead of the 15ml reached in the C growth protocol. The same rule applied for obtaining the stock solution for the resveratrol-fed (2075µg of resveratrol g^{-1} chironomid) R growth diets. (See methods section of Chapter 2 for an explanation for the differences between the C and R growth regimes with regard to their stock solution concentrations).

A total of 250µl of the relevant stock solutions described above were then added to a 3 gram aliquot of thawed chironomid larvae according to the diet treatment being made. These stock solutions were mixed gently with the chironomid larvae and left to soak in the dark for 2 hours at 4°C.

Meanwhile, a gelatine solution was prepared. Gelatine powder (5g) from bovine skin (Sigma-Aldrich) was added to 200ml of Millipore-water and dissolved at 50°C for 30 minutes on a magnetic heater set on a low mixing speed to avoid bubbling. The gelatine solution was left to cool for at least one hour as high temperatures can inactivate resveratrol. 1ml of this gelatine solution was then added to each of the chilled 3g enriched aliquots of chironomid larvae. To prevent degradation, the cubes were then freezed at -20°C until use.

APPENDIX II

Analysis of both baseline chironomid larvae and lutein enriched larvae by HPLC

High-performance liquid chromatography (HPLC) analysis was carried out in order to confirm that chironomid larvae that had not been experimentally enriched showed no signs of artificially added antioxidants. Alongside this, the HPLC analysis was carried out to determine how well carotenoids were retained in the chironomid larvae that were made following the protocol developed above in Appendix I. The chironomid larvae samples that were used to test this were frozen and then thawed in water before the HPLC analysis. This was carried out in order to imitate the experimental feeding procedure so that lutein retention in the chironomid larvae could be accurately determined.

An adapted protocol by Hess et al. (1991) was used as a reference to extract carotenoids from the samples of a) high carotenoid enriched chironomid larvae (See Appendix 1 for preparation methods) and b) non-enriched chironomid larvae. Ethanol (1000µl) and distilled water (500µl) were added to each sample of chironomid larvae (0.25g). This step extracts both free and protein-bound carotenoid molecules (Hess et al. 1991). Each sample was then vortexed for 20 seconds. In order to extract any carotenoids from the chironomid mixture, 900µl of this mixture was added to hexane (700µl) and vortexed for a further five minutes. The mixture was then centrifuged for 10 minutes (13,600 rpm, 4°C). The hexane layer (which would contain any carotenoids that had been present in the chironomid larvae sample) was then removed (the top layer) and dried under a stream of nitrogen, N_2 gas in a fume cupboard. A total of 200µl of ethanol and acetonitrile (1:4) was added to dissolve the dried hexane extract. Next, the sample was centrifuged for a further 10 minutes (13,600 rpm, 4°C) and was then ready to be injected through the HPLC system. See Figure II for the HPLC chromatogram of the chironomid larvae extracts at 440nm.

Lutein content in the processed chironomid larvae samples were quantified using a Thermo Scientific Spectra SCM1000 liquid chromatography system with a Synergi 4µ Hydro-RP reverse-phase column (length 250mm, internal diameter 2mm; 4µm particle size; Phenomenex, Macclesfield, UK). The mobile phase was HPLC grade methanol (100%, A) and ethyl acetate (97.5%, B) in gradient elution (0 to 50% B from 0-20 min), a flow rate of 0.2ml min^{-1} and detected using a diode array detector scanning between 250nm and 600nm. Chromatogram peak areas were quantified with Thermo Scientific Xcalibur

software at 440nm. The lutein peak in the chironomid larvae samples were identified and quantified in comparison with a lutein reference standard (LGC standards, Middlesex) using the same HPLC conditions.

The lutein reference standard had a mean apex retention time of 5.8 minutes and was identified as a peak with absorption maxima at 440nm. Figure I depicts the results of duplicate 5µl samples of 5, 25 and 50ng lutein standard dilutions. The standard eluted as one peak and the area of the peak was plotted against the amount of lutein standard injected into the chromatography system. This regression line was then used to calculate the concentration of lutein in the chironomid larvae samples to ascertain how well lutein was retained. The linear regression equation y = 46682x + -5513.6, where y = the peak area of lutein in the chironomid larvae extracts at 440nm was used to calculate this.

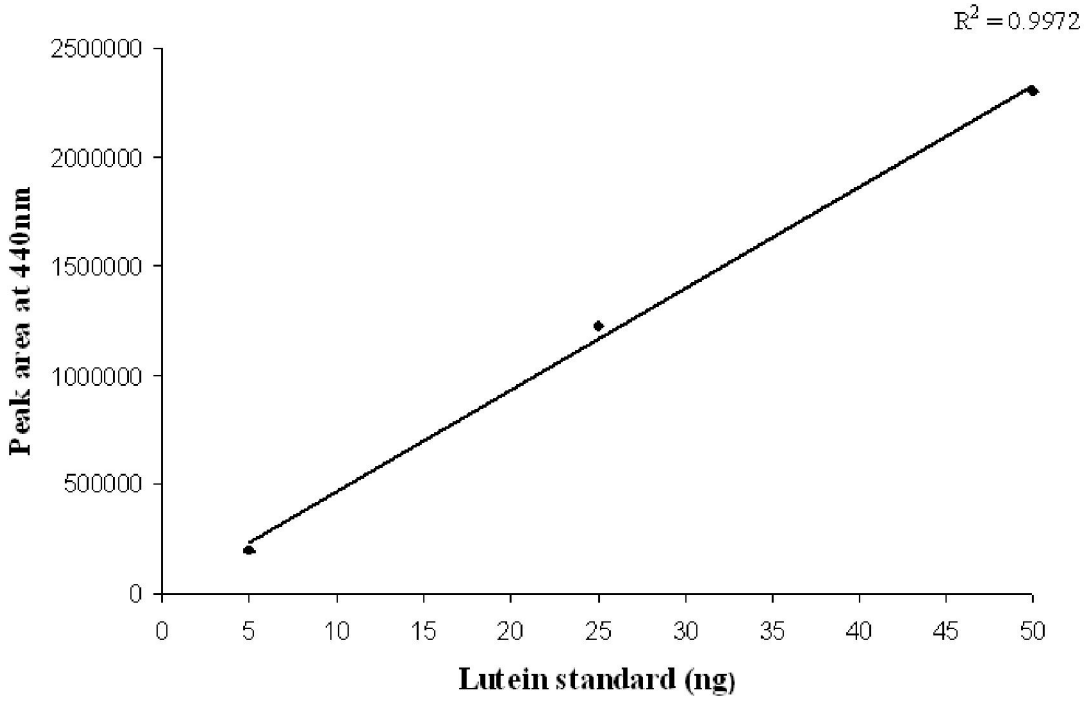


Figure I Regression line generated from standard dilutions of 5, 25 and 50ng of lutein standard per μL^{-1} of methanol.

The HPLC chromatogram illustrates that lutein was retained in the high carotenoid enriched food after being thawed in water (Figure II, red line). This chironomid sample enriched with lutein had an apex retention time of 5.9 minutes (Figure II, red line). Using

the linear regression equation described above, the lutein content in the 5µl injected sample was extrapolated to calculate how much lutein was retained per gram of thawed high carotenoid enriched chironomid larvae. This was found to be 325.67ng of lutein. To achieve 100% extraction efficiency the high carotenoid chironomid sample should have retained 375ng of lutein. Therefore, the extraction efficiency was 87%. This value was used to correct for the loss of lutein during the experimental feeding procedure. Therefore, by accounting for this loss, a more accurate estimate could be made of the total lutein content received by the fish.

The baseline chironomid larvae had a lutein content of 8.3ng and an apex retention time of 6 minutes (Figure II, black line). This lutein content is only 2.5% of the concentration found in the high carotenoid enriched chironomid larvae and therefore it can be concluded that although lutein can be detected in the non-enriched chironomid larvae, it is negligible. It can also be seen from the HPLC chromatogram that no other potentially confounding carotenoids were found in the non-enriched chironomid larvae as there was an absence of absorbance peaks at 440nm. If there had been baseline levels of carotenoids within the non-enriched chironomid larvae, absorbance peaks would have been seen in the chromatogram. For instance, alongside lutein and astaxanthin, β-cryptoxanthin, zeaxanthin, β-carotene and violaxanthin are all carotenoids that have absorption maxima in the range 400-440nm but were absent from the chromatogram (Mercadente et al. 1997).

The results of this HPLC analysis validate the food preparation protocol adopted in this study (described in Appendix I). Lutein was well retained in the enriched food after being thawed in water. The HPLC analysis also validated that the baseline non-enriched chironomid larvae contained no artificially added carotenoids which may have confounded the results produced in this study.

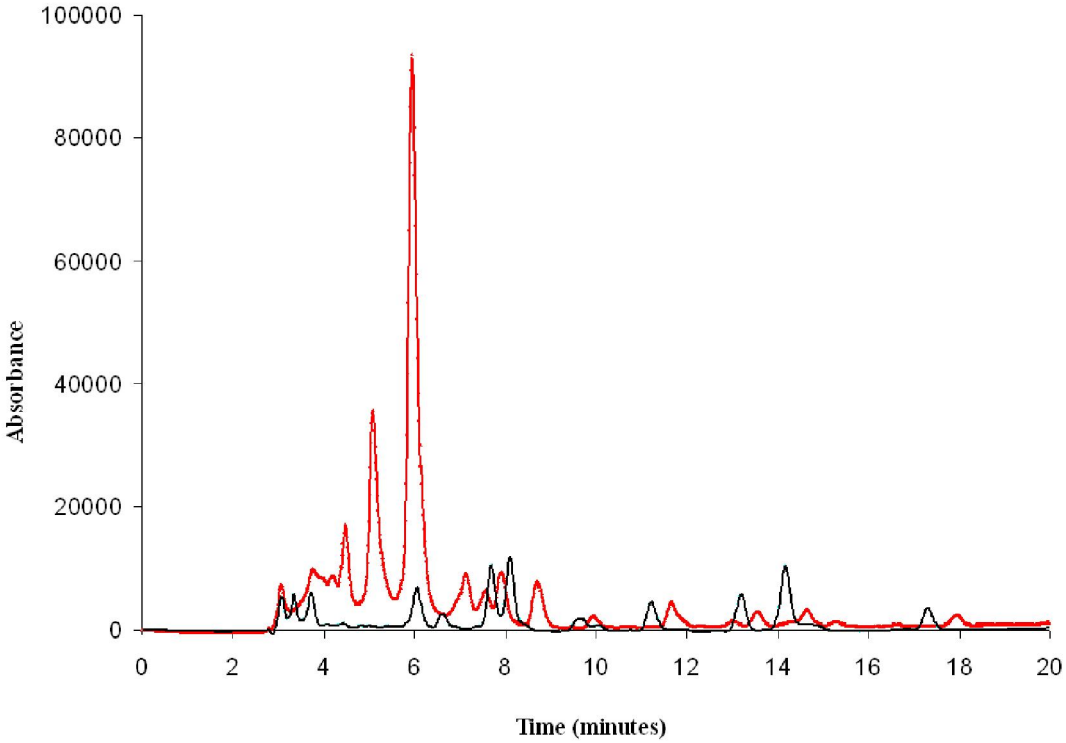


Figure II HPLC chromatogram of the chironomid larvae extracts at 440nm (High lutein carotenoid enrichment – red line and no enrichment – black line.

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