

# Consistency of behaviours over time and context in zebrafish

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# Abstract

Animal personality is defined as individual variation in behaviour which is consistent over time and across contexts; the five major components of personality are activity, boldness, exploration, sociability, and aggression. Many taxa show sex differences in behaviours, including in personality. Most studies do not assess repeatability of behaviour, and if they do, they generally assess repeatability over only relatively short time periods (e.g., a week or a month). Understanding whether personality traits are repeatable over longer periods is important for the assessment of individual differences in behaviour; if a behaviour is not repeatable over time, then it is uninformative about consistent behavioural syndromes within individuals. A core notion of personality research is that behaviours are consistent in different contexts, but there is also evidence of behaviour changing within individuals with a change in certain contexts. Thus, understanding which contexts cause a change in behaviour and which do not may be important for future research that assumes consistency of behaviour. In this thesis, I use zebrafish (*Danio rerio*) as a model to assess behavioural consistency over time and context.

In Chapter 2, I investigate the repeatability of activity, exploration, boldness, anxiety, and sociability to obtain longer-term (6 month) estimates of repeatability. I hypothesised: that these behaviours would be repeatable; that there would be sex differences both in

repeatability estimates and in actual values; and, finally, that behavioural syndromes would be expressed. Specifically, this required that the expression of one personality trait would be correlated with another. To answer these questions, individuals were filmed in a test sequence, comprising a novel arena assay (to test activity, anxiety and exploration) and a novel object assay within the same tank (to test boldness). Single-sex groups were then filmed in a different arena to test sociability. Behaviours were quantified using the behavioural analysis programs EthoVision XT and idTracker. Overall, activity and exploration in individuals, and sociability at the tank level, were fairly repeatable over six months. Boldness was also repeatable, but to a lesser degree. A commonly-used measure of anxiety was more repeatable in the novel arena assay than the novel object test. There were clear sex differences in aspects of activity and sociability, in that males were more active and shoaled less tightly than females. However, while males were more repeatable in exploratory behaviour, there were minimal sex differences in the mean expression of exploration and boldness. There was evidence for an activity-boldness-exploration behavioural syndrome, but conclusions drawn from this part of the analysis are tentative because of the non-independence of the activity and exploration data.

After demonstrating that anxiety over the first ten minutes in a novel tank is a repeatable behaviour in zebrafish, in Chapter 3 I assessed how a behavioural challenge, like social isolation, affects anxious behaviour. In social animals, isolation from conspecifics can negatively affect both behaviour and physiology. It has been previously demonstrated that isolating zebrafish from conspecifics can induce them to become more anxious and aggressive, but I aimed to investigate whether individuals that were more or less anxious than the norm would react differently, and whether their anxiety levels would remain similar to their initial behaviour. I predicted that, after a three-week period of isolation, initially non-

anxious zebrafish would become more anxious, and anxious fish would remain so. Using only the extreme anxious and non-anxious fish, I kept each individual either isolated or in a group of five for a three-week period, and then phenotyped these same fish again to get a post-treatment estimate of anxiety. I found that all four treatment groups (anxious or non-anxious, and isolated or grouped) showed a mean change in anxiety, but while the anxious isolated and group-housed fish remained on the anxious side of the spectrum and the non-anxious group-housed fish remained on the non-anxious side, the non-anxious isolated fish showed double the change of any other group, and were (on average) classed as mostly anxious after the treatment. My sample sizes were small, and so my model did not detect an overall significant change, but the effect sizes indicated a true effect of treatment despite the low power of my study. This supports part of my hypothesis that non-anxious fish would become more anxious after a period of isolation.

The major findings from this study were that activity- and exploration-related (and, to a lesser degree, boldness-related) behaviours were repeatable over a six-month period, and that social isolation is enough of a behavioural challenge to alter previous anxiety levels, especially in initially non-anxious fish. I also found evidence of both sex differences and behavioural syndromes over time. The research done in this thesis contributes to the established literature by generating long-term behavioural repeatability estimates for a model species, as well as an experimental assessment of how social isolation can alter anxiety levels. Future research should evaluate the applicability of behavioural tests to the behaviours they intend to study.

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# Chapter One

## General Introduction

Behaviour in animals differs not just among species and populations, but also among individuals. Animal personalities have been observed in many vertebrate taxa, including fish, birds, rodents, and lizards (Koolhaas et al. 1999; Cote and Cloert 2007; Sih and Bell 2008; Cote et al. 2010; Brust et al. 2013; Martins and Bhat 2014). Personality is generally defined as individual consistency in behaviours over time and across contexts and situations (Dall et al. 2004; Sih et al. 2004a; Réale et al. 2007; Poulin 2013). In behavioural ecology, context is defined as a functional behavioural category such as courtship, foraging, or dispersal, while a situation is defined as the current conditions at a point in time (Sih et al. 2004a; van Oers et al. 2005). A situation may involve different conditions in time, such as breeding seasons, or it could be different places along an environmental gradient, such as availability of food or level of predation risk (Sih et al. 2004a). However, “context” is often used to describe overall changes in both context and situation.

There are many different ways of discussing behavioural traits, but Réale et al. (2007) proposed five major categories of personality: boldness-shyness, exploration-avoidance, activity, sociability, and aggressiveness. Boldness-shyness relates to an individual’s reaction

to a risky situation, including territory defence or response to a predator. Exploration-avoidance is response to a new situation, including new habitats or food sources. Activity is an individual's general activity level, which is constrained by metabolic costs (Sih et al. 2004b), and can be associated with exploratory and shoaling behaviour in fish (Budaev 1997b; Budaev 1997a; Moretz et al. 2007). Sociability is defined as the degree to which an individual seeks or avoids the presence of conspecifics, while aggressiveness describes the degree of antagonism in interactions with conspecifics (Réale et al. 2007). A further behaviour of interest, although not included as one of the “big five” personality traits, is anxiety. Anxiety is generally defined as anticipatory fear, where there is an uncertain potential threat, but no certain or immediate risk (Belzung and Philippot 2007; Maximino et al. 2012).

## **1.1 Different behavioural types employ different strategies**

Understanding personality traits is important because consistent individual differences in behaviour are often heritable and related to fitness (Lesch et al. 1996; Dingemanse et al. 2004; Réale et al. 2007; Biro and Post 2008). Risk management is a major driver of behavioural evolution (Lima and Dill 1990; Brown et al. 2014). Prey species must balance the timing of their response to predation risk with the need to forage and find mates (Lima and Dill 1990). There are different strategies to deal with risk, each of which carries its own advantages and drawbacks. Total plasticity of behaviour would be the most adaptive scenario – for example, an individual can be bold when this is advantageous, such as for obtaining resources, but it can also be more cautious when needed, such as when waiting for a predator to move away before emerging from shelter (Lima and Dill 1990; Dale et al. 2001; Sih et al. 2004a; Bell et al. 2009). However, limited within-individual plasticity, where an individual consistently displays a particular type of behaviour across situations, is common in the

animal kingdom (Sih et al. 2004a; van Oers et al. 2005; Bell et al. 2009; Dingemanse et al. 2012; Dochtermann and Dingemanse 2013). Thus, while strategies differ within populations, individuals tend to be consistent in the way they manage risk (Brown et al. 2014). For example, an individual might be consistently exploratory even when this is inappropriate, and thus make itself vulnerable to predation or other potential hazards in a novel environment (Sih et al. 2004b).

Behavioural syndromes describe across-individual linkage of different personality traits, such as boldness and exploration (Sih et al. 2004a; Dingemanse et al. 2012). In Steller's jays (*Cyanocitta stelleri*), risk-taking (measured as alarm calling) and exploration (measured as distance travelled outside home territory) are correlated (Gabriel and Black 2010). Correlations between sociability, boldness, and exploration were found in a study of factors influencing dispersal in mosquitofish (*Gambusia affinis*); individuals with low sociability but high boldness and exploration scores were more likely to disperse along an artificial stream (Cote et al. 2010). Convict cichlids (*Amatitlania nigrofasciata*) that are fast to explore are slower to react to a model predator (Jones and Godin 2010). Personality-related behaviours, and thus behavioural syndromes, can be quantified using established methods of behavioural testing.

## 1.2 Behavioural tests

Exploratory behaviour is commonly tested using open field tests, or modified versions of such, where the behaviour of an individual in the arena is recorded over a period usually of five to ten minutes (Archer 1973; Dingemanse et al. 2002; Herde and Eccard 2013; Thomas et al. 2016). Open field tests were originally developed for use with rodents but are now used to study exploratory behaviour in many other taxa; they are also referred to as novel arena

tests (Archer 1973; Herde and Eccard 2013; Menzies et al. 2013; Germano et al. 2017).

Novel arena tests are also used to quantify activity, often as total distance travelled or mean velocity (Boon et al. 2007; Montiglio et al. 2010; Dziewczynski and Crovo 2011; Schuster et al. 2017). Rate of activity is positively correlated with rate of food intake and productivity in a range of taxa (reviewed by Biro and Stamps (2008)). In red squirrels (*Tamiasciurus hudsonicus*), the mother's levels of activity in the nest affect offspring growth, but the direction of this effect depends on food availability in a given year (Boon et al. 2007).

Boldness is often measured using latency to leave a refuge, or response to a novel object or predator model (Frost et al. 2007; Harris et al. 2010; Raoult et al. 2012; Germano et al. 2017). However, a comparison of study methods in wild baboons (*Papio ursinus*) indicated that responses to a threat (here, a venomous snake) are different from responses to a non-threatening novel object, and that threat-related behaviours may be more a measure of anxiety than boldness (Carter et al. 2012). In animals captured from the wild, boldness is heavily influenced by the level of predation in the home range of the population, with areas of higher predation producing consistently bolder individuals (Brown et al. 2005). There is also a documented link between boldness and stress response in teleosts, where bolder individuals react differently to stress than shyer individuals (Brown et al. 2005; Thomson et al. 2011; Raoult et al. 2012).

There are also many ways of testing anxiety, but this behaviour is generally tested in the most appropriate method for a given organism. In pelagic fish, this is usually a novel tank diving test, where the proportion of the first few minutes in a novel tank spent in the bottom part of the tank is an indicator of anxiety (Egan et al. 2009; Maximino et al. 2012; Parker et al. 2012). In zebrafish, an average fish is expected to spend about 55% of the first five minutes

in a novel tank at the bottom (Levin et al. 2007). Anxiety is generally considered to be the fear of potential, rather than immediate, danger (Belzung and Philippot 2007; Maximino et al. 2012). There is a known link between anxiety and activity behaviours; an alarm reaction in shoaling fish involves both increased time at the bottom of the tank, and fast, erratic movement (Speedie and Gerlai 2008; Egan et al. 2009; Gerlai 2013).

Sociability is often tested by separating the focal individual from a group of conspecifics while still allowing visual contact, and recording time spent near or away from the group (Cote et al. 2010; Brodin et al. 2013). In fish, sociability can be tested by measuring shoaling behaviour, where software is used to analyse videos of a group swimming together, to quantify group cohesion (Miller and Gerlai 2007). Two commonly-seen effects of being in a social group (as opposed to being isolated) are conformity and facilitation (Ward 2012; Herbert-Read et al. 2013). Where there is a conformity effect, individual variation in behaviour is suppressed by the group, and each individual's behaviour becomes more similar to the group mean (Herbert-Read et al. 2013). A facilitation effect increases the frequency of expression of a risky behaviour such as exploration or foraging (Ward 2012). An example of the effect of a social group is that, in zebrafish, behaviour is less variable when tested at the level of a small group than at an individual level (Pagnussat et al. 2013).

### **1.3 Behavioural repeatability**

Given that personality is defined as behaviour that is consistent in individuals over time and across contexts (Réale et al. 2007), it is important to assess whether any trait being measured is repeatable. A repeatable behaviour is one where within-individual variation is smaller than between-individual variation (Dingemanse et al. 2012). Bell et al. (2009) performed a meta-analysis of repeatability of a wide range of behaviours across many taxa, and found strong

support overall for the hypothesis that behaviour is repeatable. Most of the studies included in their analysis examined courtship and mate preference behaviours; only six studies out of 114 quantified the repeatability of activity and exploration, and there were no studies which tested boldness. A more recent survey of the literature for boldness, activity, and/or exploration, from 2002 to 2017, demonstrates that repeatability of behaviour ranges from -0.03 to 0.68, with a mean of 0.37 and standard error of 0.02 (Table 1). Many of the studies performed repeated measures of behaviour over very short periods; the shortest was in Rudin et al. (2016), where crickets were re-tested within a single day, while the longest was in Wilson and Godin (2009), where sunfish were tested up to three months after the initial tests. These studies also used a variety of testing methods to study these behavioural traits. Some tests need to be altered to be appropriate for the animal being tested, but there are also multiple methods that are commonly used for testing the same behaviours; their applicability to the trait in question can be a matter of debate (Burns 2008), and even the design of a test arena can affect the outcome of a behavioural test (Näslund et al. 2015). For example, the novel environment emergence test, where latency to leave a shelter is recorded, has been used to test boldness and exploration separately, but it has been suggested that it may instead be testing the conflict between shyness and exploratory tendency (Burns 2008; Näslund et al. 2015).

A clear issue with some of the studies in Table 1.1 is that of sample size. Most of the studies listed had adequate sample sizes, ranging from 30 to 326, but Wilson and Godin (2009) and Carlson and Langkilde (2013) both used very small samples, of 13 and 8, respectively. Both of these studies also reported repeatability values that were near the extremes of the range of values from all the studies in the Table; their small sample sizes may have contributed to this. A third study with a similarly small sample size of 17, however, reported a repeatability

estimate (0.30) that was closer to the table mean of 0.37 (Byrnes and Brown 2016). Moreover, there were few studies that reported repeatability values for males and females separately, despite several studies finding sex differences in behavioural repeatability (Dingemanse et al. 2002; Schuett and Dall 2009; Hedrick and Kortet 2012; Tran and Gerlai 2013). Overall, the studies presented in Table 1.1 demonstrate that further research into the repeatability of behaviours, with adequate sample sizes and over longer time periods, is needed.

*Table 1.1:* A sample of repeatability estimates (R) for boldness, exploration, and activity found in the literature. Test intervals are the time between measurements of behaviour, and Sig. is whether the repeatability estimate was significant (y) or non-significant (n). n is number of individuals that were tested repeatedly. OFT stands for open field test, and NO stands for novel object.

Common name	Trait	Test method	R	Test intervals	Sig.	Sex	n	Author(s)
Red squirrel	Activity	OFT	0.41	6 weeks	y	Female	55	Boon et al. (2007)
Little brown bat	Exploration	Novel environment test	0.29	24 hours	y	Both	76	Menzies et al. (2013)
	Activity		0.34		y	Both	76	
Eastern chipmunk	Activity	Distance travelled in OFT	0.3	≥ 15 days	y	Both	81	Montiglio et al. (2010)
House mouse			0.59		y	Both	326	
Harvest mouse	Boldness	Proximity to wall in OFT	0.27	7 days	y	Both	38	Schuster et al. (2017)
	Exploration	Interaction with NO	0.39		y	Both	39	
	Activity	Distance travelled in OFT	0.45		y	Both	38	
Gray mouse lemur	Exploration	OFT	0.25	≥ 14 days	y	Both	72	Thomas et al. (2016)
Great tit	Exploration	Novel environment test	0.48	≥ 7 days	y	Male	111	Dingemanse et al. (2002)
			0.27		y	Female	74	
Desert tortoise	Boldness	Fear response to predator model	0.41	≥ 7 days	y	Both	59	Germano et al. (2017)
	Exploration	Novel environment test	0.21		n	Both	59	
Keelback	Boldness	Latency to emerge (head)	0.39	2-7 days	y	Both	40	Mayer et al. (2016)
		Latency to emerge (body)	0.45		y	Both	40	

American bullfrog (tadpoles)	Boldness	Proximity to wall in OFT	0.25	24 hours	n	Both	8	Carlson and Langkilde (2013)
	Exploration	Gridlines crossed in OFT	0.68		y	Both	8	
	Activity	Proportion of time moving	0.12		n	Both	8	
Zebrafish	Exploration	OFT	0.4	7 days	y	Male	30	Toms and Echevarria (2014)
	Boldness	Latency to approach NO	-0.03		n	Male	30	
Three-spined stickleback	Activity	Novel environment test	0.52	2 months	y	Both	30	Dziewczynski and Crovo (2011)
Guppy	Boldness	Latency to emerge from shelter	0.34	24 hours	y	Male	61	Harris et al. (2010)
			0.29		y	Female	106	
Guppy	Exploration	Percent of tank area covered	0.46	μ 6.5 weeks	y	Female	32	White et al. (2016)
	Activity	Distance travelled	0.31		y	Female	32	
Bluegill sunfish	Boldness	Latency to emerge from shelter	0.63	1-3 months	y	Both	13	Wilson and Godin (2009)
	Activity	Time spent moving	0.15		n	Both	13	
Port Jackson shark	Boldness	OFT emergence	0.3	3 days	y	Both	17	Byrnes and Brown (2016)
Field cricket	Boldness	Latency to emerge from shelter	0.28	Not stated*	y	Female	82	Hedrick and Kortet (2012)
Australian field cricket	Boldness	Latency to emerge from shelter	0.52	Within 1 day	y	Male	116	Rudin et al. (2016)
	Exploration	Time near shelter	0.44		y	Male	88	
	Activity	Distance travelled and velocity	0.47		y	Male	88	

\* The Hedrick and Kortet study tested cricket nymphs and then the same individuals 14 days after moulting as adults, but did not state the total time elapsed between tests

## 1.4 Changes with context

While personality-related behaviours are often repeatable, there are certain contexts and challenges which can change the expression of these behaviours. Winning or losing a fight, or even watching a conspecific win or lose a fight, can affect a focal individual's subsequent level of boldness; a bold rainbow trout (*Onchorhyncus mykiss*) will exhibit shyer behaviour toward a novel object after losing or watching a conspecific lose a fight (Frost et al. 2007). Similar effects of contest wins and losses on boldness and exploration are seen in Australian field crickets (*Teleogryllus oceanicus*; Rudin et al. 2016). Social context can alter exploratory behaviour, just as individual level of exploration can influence response to social situations. For example, mosquitofish (*Gambusia holbrooki*) are more exploratory when in a larger group (Ward 2012), while male great tits (*Parus major*) which are slower to explore are more responsive to the behaviour of nearby conspecifics than faster-exploring males (van Oers et al. 2005). Social stress caused by conflict with a dominant individual can also reduce levels of activity, aggression, and reward response in subordinate individuals (Blanchard et al. 2001). On the other hand, a study on jumping spiders (*Eris militaris*) found that, while low level exposure to an insecticide did not produce a population-level effect on behaviour, it did reduce consistency of behaviour within individuals (Royauté et al. 2015). Thus, challenges can alter both within-individual and between-individual variation. This is a further indicator that it is important to study how usually consistent behaviour might change after a challenge, and to elucidate the specific effects on both individuals and populations.

## 1.4 Study species

Fish are a popular model for studying behaviour (Spence et al. 2008; Norton and Bally-Cuif 2010; Kalueff et al. 2013). Fishes have similar brain structure to other vertebrates; the

conservation of many aspects of brain function within vertebrate taxa means that research carried out on the brains and behaviours of fish is highly applicable to other vertebrate groups (Goodson 2005; Bshary et al. 2014). More specifically, the zebrafish is a common model organism in brain and behaviour research (Speedie and Gerlai 2008; Steenbergen et al. 2011; Teles et al. 2013), and so there is a wealth of information about this species. Kalueff et al. (2013) developed a comprehensive catalogue of behaviours exhibited by zebrafish across different situations, including reproductive, pain-related, and reward-related behaviours. Zebrafish have also been used to answer a wide range of personality-related questions (Miller and Gerlai 2007; Norton and Bally-Cuif 2010; Steenbergen et al. 2011; Butail et al. 2013; Cianca et al. 2013; Gerlai 2013; Tran and Gerlai 2013; Bshary et al. 2014; Holley et al. 2014; Zajitschek et al. 2017). Their short generation time and ease of husbandry make zebrafish ideal candidates not only for behavioural research but also for genetic and neurological studies (Norton and Bally-Cuif 2010). Zebrafish are also a reliable model for behavioural, pharmacological, endocrine, and genetic aspects of anxiety in humans (Egan et al. 2009; Parra et al. 2009; Norton and Bally-Cuif 2010; Gerlai 2013).

Most strains of zebrafish have been in captivity for numerous generations, and there is some suggestion that this might have resulted in selection for high reproductive capacity, but not for other physical or behavioural traits (Robison and Rowland 2005; Spence et al. 2008). Zebrafish were first described in the wild in north-eastern India. Their natural range stretches into Pakistan and Nepal in the north, and Bangladesh in the east (Spence et al. 2006; Engeszer et al. 2007; Parichy 2015). Typically, zebrafish live in small, slow streams, rice paddies, and still seasonal pools with silt or gravel on the bottom (Spence et al. 2006; Engeszer et al. 2007). Little research has been carried out on zebrafish behaviour in their natural habitat, but shoaling has been observed both in laboratory and wild situations

(Parichy 2015). Laboratory studies comparing wild-caught zebrafish from different locations (as well as domesticated laboratory strains) have shown behavioural syndromes which differ both between wild populations and between domesticated strains (Robison and Rowland 2005; Moretz et al. 2007; Martins and Bhat 2014). For example, aggression and boldness are strongly linked across wild zebrafish populations when measured in a laboratory context (Martins and Bhat 2014), but also among laboratory strains (Moretz et al. 2007). There is evidence of a domestication effect on behaviour in zebrafish, where certain laboratory strains recover from stress more quickly and are less aggressive than wild-caught zebrafish, but this effect is not seen in all laboratory strains (Moretz et al. 2007).

There has been some research on behavioural syndromes, but repeatability has seldom been studied in zebrafish. One study examined exploration and boldness behaviours over 7 days, and found that, while the measure of exploration was repeatable (0.4), boldness was not (Toms and Echevarria 2014). Another study quantified the repeatability of aggression and found that three of five behaviours were significantly repeatable (ranging from 0.096 to 0.721; Way et al. 2015). A third study by Tran and Gerlai (2013) reported that activity was consistent over 7 days in zebrafish, but the study used different methods for analysis and so did not provide a repeatability estimate. In light of this lack of published information on the subject, there is a clear need for further studies of repeatability over time to be performed using zebrafish, in order to further augment the utility of this already valuable model organism.

## 1.5 Thesis aims

The overall aim of this thesis was to examine consistency in behaviour over time, and how behaviour changes across context, using zebrafish as a model. In Chapter 2, I aimed to obtain

long-term repeatability estimates for behaviours related to activity, exploration, and boldness, as well as to identify sex differences and any behavioural syndromes that were present. I hypothesised that these behaviours would all be significantly repeatable over 27 weeks, that sex differences would be present both in behaviours and in their repeatability, and finally that there would be an activity-exploration-boldness behavioural syndrome.

In Chapter 3, I aimed to determine whether anxiety, shown to be a repeatable activity-related behaviour in Chapter 2, would alter with a change in context, and whether the magnitude or direction of differences depended on initial anxiety levels. To do this, I first phenotyped individuals as anxious or non-anxious, and then kept them isolated or in small groups for three weeks. After the test period, individuals were phenotyped again to determine whether social isolation would disrupt the expression of anxiety; I hypothesised that social isolation would change anxiety levels, with the prediction that the non-anxious isolated fish would become more anxious, while the anxious isolated fish and the fish housed in groups would show no major changes.

# **Chapter Two**

## **Long-term repeatability of behaviours**

### **2.1 Introduction**

The field of animal personality research has seen huge growth over the last two decades (Gosling and John 1999; Dall et al. 2004; Bell et al. 2009; Zajitschek et al. 2017). What was originally thought to be non-adaptive variation around an adaptive population mean is now considered to be an important aspect of fitness (Dingemanse et al. 2002; Dall et al. 2004; Smith and Blumstein 2008; Cote et al. 2010). In short, personality is defined as within-individual consistency in behaviour over time and across contexts (Cloninger 1986; Dall et al. 2004). It is clear to any observer that humans show inter-individual differences in behaviour and temperament, but it has become increasingly obvious that this is also true of non-human animals (Gosling and John 1999; Dall et al. 2004; Sih et al. 2004a). Personality is an important factor in both evolution and ecology because it may affect the way individuals respond to different selection pressures (Dall et al. 2004). For example, in a fishery, individuals with a higher growth rate, and thus greater levels of feeding activity and boldness, have more encounters with fishing equipment and are selectively removed from the population (Biro and Post 2008). Personalities may also be maintained by individuals

specialising in different social niches in order to avoid conflict with conspecifics; an example of this is that taking a submissive role might reduce an individual's ability to monopolise a resource, and thus necessitate a higher level of exploration in order to find food (Bergmüller and Taborsky 2010).

Sex differences in behaviour are commonly observed in animal populations (e.g., Lehmann and Boesch (2008) and Schuett and Dall (2009)). Males are often bolder and more aggressive than females; this has been shown in fish and birds, but less extensively in other taxa (Schuett and Dall 2009; Harris et al. 2010; Dahlbom et al. 2011; Carazo et al. 2014). There are, of course, exceptions; female spotted hyenas are dominant over males, and tend to be more aggressive than males (Gosling 1998). Female mice, however, tend to be both more anxious and more defensive in the face of a potential threat than males (Blanchard et al. 1991; Palanza 2001). In primates, one sex tends to disperse at maturity, while the other shows philopatry (Lehmann and Boesch 2008; Silk et al. 2009). It is generally the philopatric group which shows greater sociality (Silk et al. 2009; Ostner and Schülke 2014), although dispersing females still tend to show fairly high levels of sociability with other females upon encounter (Lehmann and Boesch 2008). In zebrafish, both age and sex have been shown to affect the results of behavioural assays. Under 10 months of age, males show higher activity levels, while after age 22 months, females are more active (Philpott et al. 2012). There are different methods of responding to a challenge; intra- and inter-sexual selection contribute to sex differences in behaviour and morphology (Bergmüller and Taborsky 2010). There have also been differences observed between males and females in repeatability of behaviours. Male great tits showed higher repeatability of exploratory behaviour in a novel environment test than females (Dingemanse et al. 2002). Similarly, male zebra finches were more consistent in exploratory behaviour than females, despite the influence of the exploration

level of a nearby conspecific (Schuett and Dall 2009). Conversely, crickets were repeatably bold across metamorphosis in females, but not males (Hedrick and Kortet 2012), and female zebrafish were more consistent than males in activity levels over a period of seven days (Tran and Gerlai 2013). A meta-analysis by Bell et al. (2009) of mating-related behaviour studies found that males from a wide range of taxa were reported as more repeatable in mating and courtship behaviours than females.

Linkage between boldness, aggression, and exploration is common in animal populations (Koolhaas et al. 1999; Sih et al. 2004a; Réale et al. 2007). This is generally known as a behavioural syndrome (Réale et al. 2007; Dingemanse et al. 2012). Individuals showing high levels of these traits are described as proactive; they are bold, actively explore their environment, and tend to form routines. On the other side of the spectrum are reactive individuals, which are more shy and less exploratory, but pay more attention to their environment and so adapt better to new challenges (Koolhaas et al. 1999; Sih et al. 2004a). Proactive and reactive individuals show different coping strategies for stress, danger, and aversive stimuli (Koolhaas et al. 1999). Specific life-history strategies are also differently employed along the proactive-reactive spectrum; proactive individuals tend to have a higher food intake and growth rate than reactive individuals, possibly because aggressiveness and boldness may enable monopolisation of resources and thus allow high levels of growth and reproduction (Stamps 2007; Biro and Stamps 2008). Likewise, there is a link between personality and reproductive fitness (Smith and Blumstein 2008; Zajitschek et al. 2017). It has been illustrated that personality traits in great tits are quick to respond to directional artificial selection over five generations toward either proactivity or reactivity (Drent et al. 2003). This indicates that personality-related behaviours are heritable, and so, along with their relevance to mating, foraging, and dispersal, they may be major components of fitness

(Dingemanse et al. 2002; Cote et al. 2010). As in most aspects of fitness, there are trade-offs; bolder males have better reproductive success, but boldness incurs a fitness cost in that it makes individuals more vulnerable to predation (Stamps 2007; Wolf et al. 2007; Smith and Blumstein 2008). Anthropogenic effects can alter population-level frequency of personality types (Sih et al. 2004a; Biro and Post 2008). In a population of game fish, it was demonstrated that human fishing activity, by removing the proactive, faster growing individuals from the population, selected for slower-growing reactive individuals to remain within it (Biro and Post 2008). Individual personality traits may thus need to be accounted for when issues of conservation, especially captive breeding and reintroduction programmes, are discussed (McDougall et al. 2006; Réale et al. 2007).

Understanding animal personality is important to our understanding of animal behaviour and life history evolution. In order to comprehend why within-individual consistency in behaviour is maintained in wild populations, we need to know how heritable these behaviours are over generations, and to what degree they are repeatable within individuals (Dingemanse et al. 2002; David et al. 2012). Such estimates of consistency over time, measured as individual repeatability, can help contextualise behavioural research. To estimate repeatability, behaviours are measured more than once in the same individuals, and analysed to determine what proportion of variation is owing to differences between individuals, and what portion is due to variation within individuals. There are several different methods of calculating repeatability (Lessells and Boag 1987; Hayes and Jenkins 1997; Nakagawa and Schielzeth 2010; Stoffel et al. 2017), but the unifying criterion is that, in a repeatable behaviour, the variation within individuals is smaller than the variation between individuals (Bell et al. 2009). It can be assumed that phenotypic flexibility, measurement or observer error, and random error make up the non-repeatable portion of behavioural variation

(Nakagawa and Schielzeth 2010). Behaviour can vary between individuals not just according to environmental conditions, but also because of endogenous factors, such as stage in the breeding cycle (Wilson and Boelkins 1970; Husak 2006; Fallon et al. 2016), across metamorphosis (Hedrick and Kortet 2012; Wexler et al. 2016), or simply with age (Class and Brommer 2016; Dreiss et al. 2017). Repeatability of behaviour has been demonstrated in a wide range of taxa and for different behaviours (Table 1.1; see also Bell et al. (2009)), but many studies do not perform repeated tests of individuals. Repeatability estimates differ between studies, probably owing both to differences in methods and in study species. For example, activity measured in a novel arena test, an assay often used to measure activity, was highly repeatable ( $R = 0.52$ ) in three-spined sticklebacks (*Gasterosteus aculeatus*) over two months (Dzieweczynski and Crovo 2011), and in the house mouse (*Mus musculus*) over two weeks ( $R = 0.59$ ; Montiglio et al. 2010). On the other hand, exploratory behaviour measured in a similar novel environment test in the desert tortoise (*Gopherus agassizii*) was not repeatable over 7 days ( $R = 0.21$ ; Germano et al. 2017), but exploration by the great tit (*Parus major*) over the same time frame was repeatable ( $R = 0.48$ ; Dingemanse et al. 2002).

While personality has been measured in numerous animals (Gosling 1998; Brown et al. 2005; Cote and Clobert 2007; Biro and Post 2008; Schuett and Dall 2009; van Dongen et al. 2010; Royauté et al. 2015), estimates of repeatability, particularly longer term repeatability, are lacking (but see David et al. (2012)). Most studies which do estimate repeatability of behaviours only do so over short time periods of days or weeks, and only occasionally over months (see Table 1.1). Thus, the main aim of this experiment was to test whether a suite of behaviours related to activity, exploration, boldness, and sociability, as described by Réale et al. (2007), were repeatable over a 27-week period. I also wanted to determine whether there were sex differences, not just in the behaviours themselves but also in their repeatability. The

last aim of this research was to test for behavioural syndromes, because correlation of different types of behaviour is an important aspect of animal personality. Given that personality implies individual consistency in behaviour over different contexts, I predicted that the behaviours would be fairly repeatable over time. Differences in behaviour between males and females have been noted across many taxa, so I expected to see sex differences in my findings; specifically, I predicted that males would tend to be more bold, active and exploratory, and that females would be more sociable. Finally, I also predicted that personality-related behaviours would be correlated, because strong links among activity, boldness, and exploration have been demonstrated in both laboratory and wild populations in the literature.

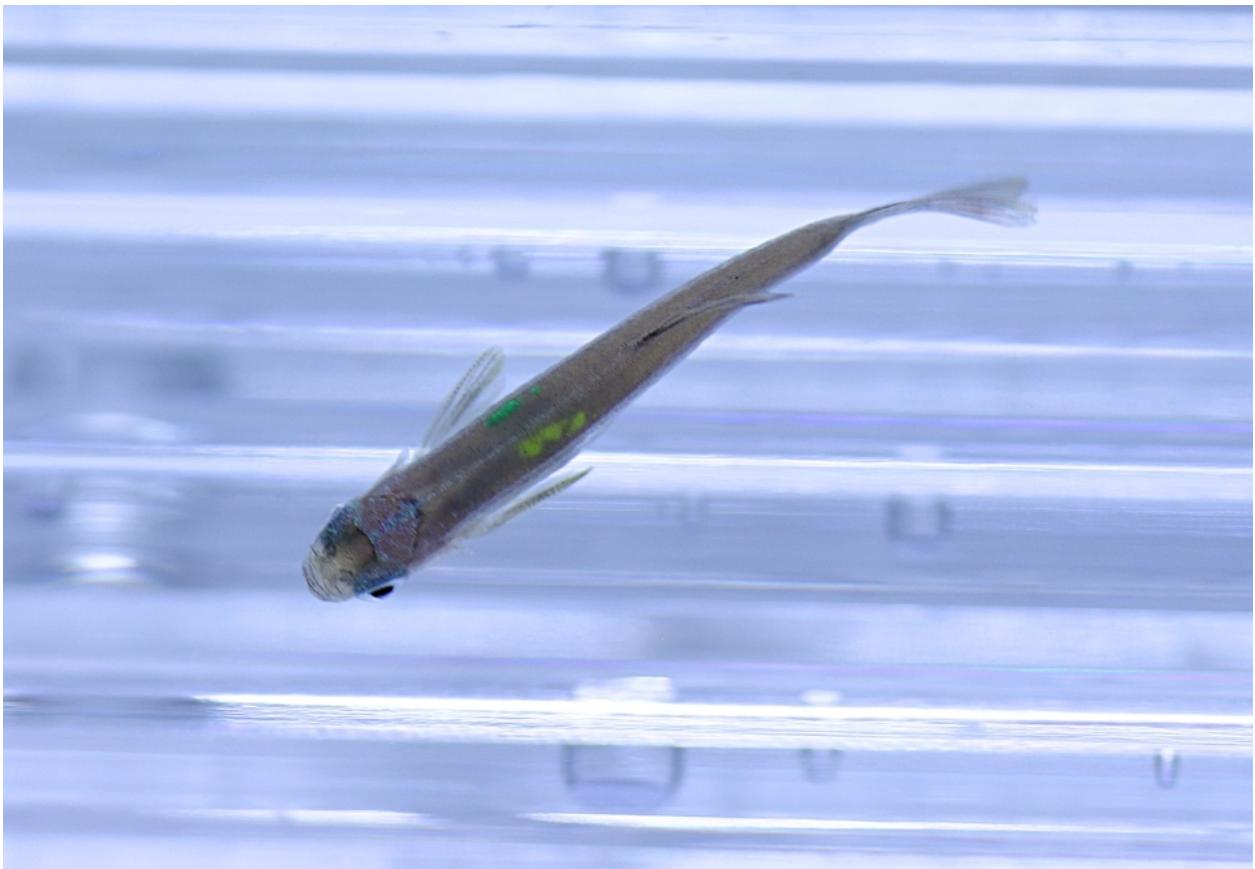
## 2.2 Materials and Methods

### 2.2.1 Subjects and husbandry

The subjects of this study were zebrafish (*Danio rerio*) bred in the Otago Zebrafish Facility. Fish were hatched on the 22<sup>nd</sup> of March 2016 and reared in a temperature-controlled facility (25-27°C) with a 08:00 to 22:00 light cycle, until they were five months old. On the 19<sup>th</sup> of September 2016, fish were moved to the Department of Zoology and held in a temperature-controlled room (25°C) with a 07:00 to 20:30 light cycle, and 30 minutes of twilight each to simulate dawn and dusk. The Techniplast holding tanks were 3.25L (284mm x 169mm x 114mm) on a custom-built rack with built-in physical and UV light filtration (Fig. 2.2.1). The sump which supplied the holding system with water was topped up from a second sump, which was filled with filtered tap water in the afternoons. This sump was aerated by air stones to remove chlorine, and was left overnight to come to the temperature of the room. On weekdays, the fish were fed three times daily; dry feed in the mornings and afternoons and a live feed of brine shrimp (*Artemia sp.*) at midday. On weekends, the fish received one dry feed and a live feed, usually both at about midday. Each fish was tagged with visible implant elastomer (VIE) tags at 4 months age, to allow identification of individuals (Fig. 2.2.2). Most fish had two tags, but some had only one, and two fish had no tags.



*Figure 2.2.1:* The system in the Zoology Department on which the zebrafish were kept. The secondary sump is shown on the bottom left (green) and the system sump is on the bottom of the trolley (blue).

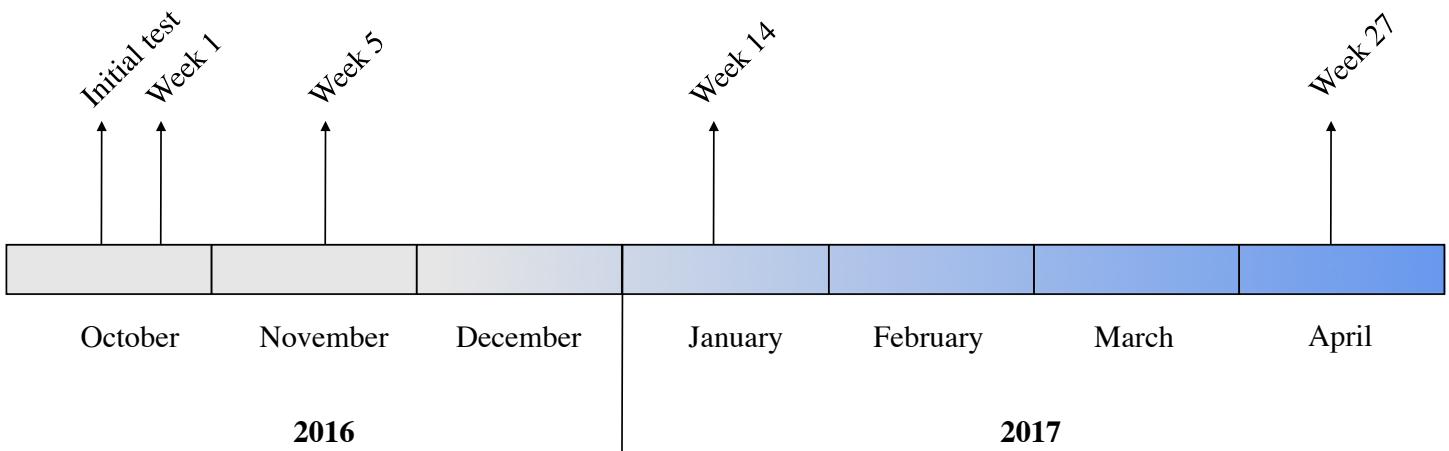


*Figure 2.2.2:* A zebrafish with fluorescent elastomer tags. Tags are read left-to-right, anterior-to-posterior, looking at the fish from above; this fish would be identified as “yellow-green”.

## ***2.2.2 Experimental protocols***

At the beginning of the experiment, there were 24 males and 24 females. Initial behavioural tests were performed on the 13<sup>th</sup> and 14<sup>th</sup> of October 2016, one-week repeat tests on the 20<sup>th</sup> and 21<sup>st</sup> of October, five-week repeat tests on the 17<sup>th</sup> and 18<sup>th</sup> of November, fourteen-week repeat tests on the 12<sup>th</sup> and 13<sup>th</sup> of January, and twenty-seven-week repeat tests performed on the 13<sup>th</sup> and 14<sup>th</sup> of April 2017 (Fig. 2.2.3). Some mortality occurred throughout the testing period, so the total sample size for analysis was fewer than 24 males and 24 females (see details below). Testing was always performed over Thursdays and Fridays. Fish were held twelve to a tank, with males and females within each tank separated by a clear divider.

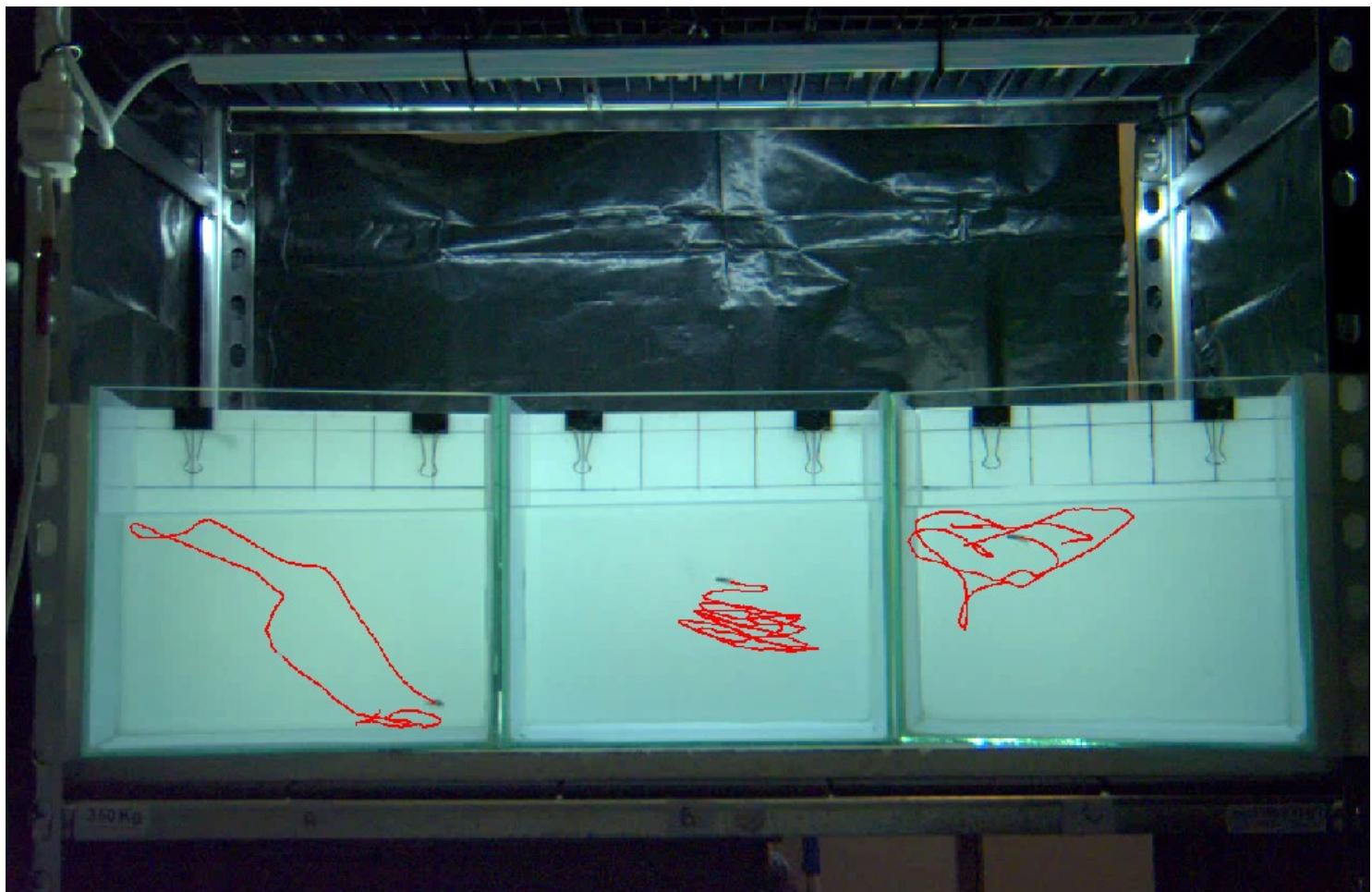
Dividers were removed after the Week 5 repeatability tests to allow the males and females to interact naturally in the tanks. Dividers were replaced on the 10<sup>th</sup> of January, two days before the beginning of the Week 14 tests. The dividers were again removed after the Week 14 tests, and finally replaced again one week before the Week 27 tests.



*Figure 2.2.3: The timeline for repeatability testing.*

Each individual fish was tested independently in a test tank (300mm H x 270mm W x 154mm D), with three tanks filmed simultaneously (Fig. 2.2.4). The tanks had two sheets of translucent white acrylic on the back outside of the tank, and were lined with white film on two sides and the bottom to eliminate glare and reflection. Tanks were lit from behind and above. The afternoon before the first test day at each time point, the test tanks were filled with half fresh sump water, and half water from the holding system so that there were chemical cues from other fish present. The reasoning behind this was that the test tanks would be swamped by chemical cues from numerous fish, rather than building up throughout the assays on a single day, since the same water was used to test up to 24 fish each day. On the afternoon of the first day of testing, half of the water was siphoned out of each tank, and the tanks were then topped back up to the same level with fresh sump water. The total volume of water in each test tank was ~10L (Fig. 2.2.5).

Behavioural analysis was performed by live-tracking using the behavioural analysis program EthoVision XT version 11.5 (Noldus Information Technology; Fig. 2.2.4). To test activity, exploration, and boldness, a sequence of two tests were performed over a 21-minute period. To control for variation in circadian rhythms, testing was carried out for each tank at the same time of day at which it was tested at the previous time points; that is, tank one and tank three fish were always tested between 09:30 and 12:00, while tank two and tank four fish were always tested between 12:50 and 15:00. On the day of testing, fish were not fed until after their tank had been tested.



*Figure 2.2.4: Live tracking using EthoVision. Each fish's swimming track over the last 30 seconds is shown in red.*



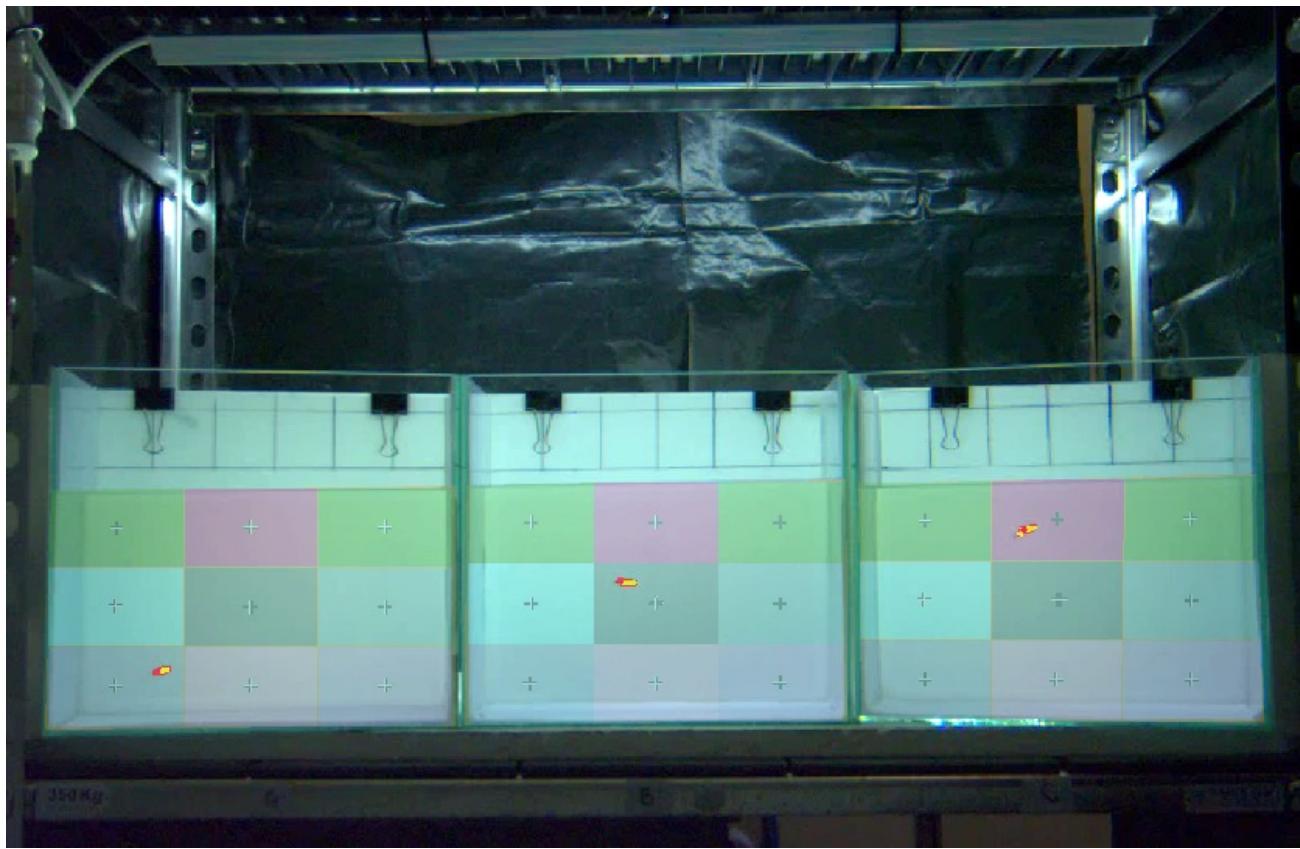
*Figure 2.2.5:* The back of the testing set up, showing the lights used to illuminate the tanks and the curtain divider to avoid the experimenter being visible to the fish. The test tanks could be accessed from the back for the insertion of the novel objects into the tanks halfway through each trial.

### **Novel arena test**

During the first ten minutes, the novel arena test assessed each individual's activity levels and exploratory behaviour. Activity was measured as the total distance travelled and mean velocity during the test, as well as the percentage of the test spent immobile. An active fish travelled far and was fast, and spent most of its time mobile. Anxiety was measured as the percentage of time spent in the bottom part of the tank. An anxious fish spent most of its time at the bottom (Fig. 2.2.6). To measure exploration, a 3x3 grid of equal-sized rectangles (70mm H x 90mm W) was superimposed, and the cumulative duration spent in each square was recorded (Fig. 2.2.7). An exploratory fish was one that explored all of the squares equally.



*Figure 2.2.6:* Tracking for activity and anxiety analysis (last 60 seconds shown in red). Total distance travelled and mean velocity are recorded, as well as percent of trial spent immobile. Fish that spend a high proportion of the trial in the bottom zone are classed as anxious.

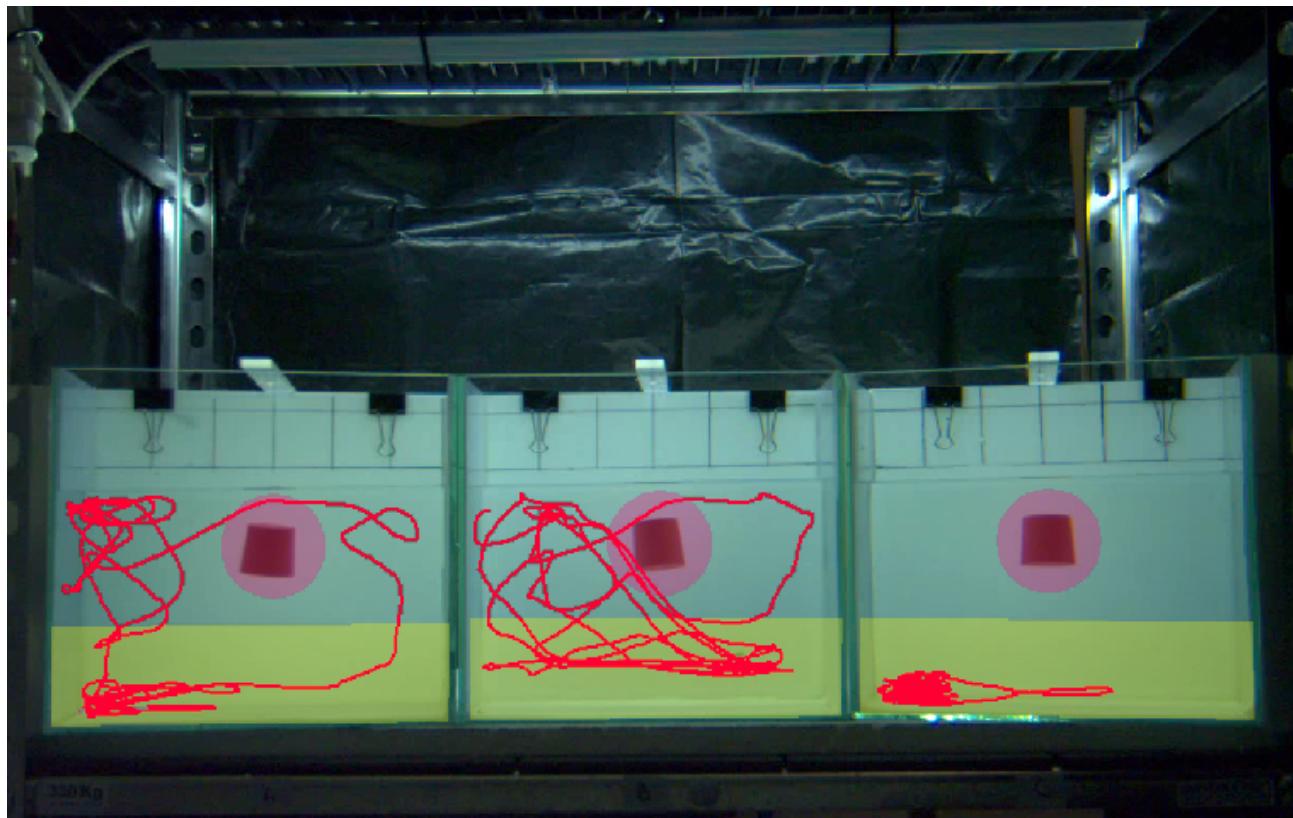


*Figure 2.2.7:* Set-up for exploration analysis. A grid is superimposed on the test tanks, and the duration and frequency of visits to each square is tracked by EthoVision. Fish are highlighted in yellow, with a red dot indicating the centre-point.

### ***Novel object test***

At ten minutes, a rubber bung hanging from nylon fishing wire (diameter 140mm, height 38mm) was placed into the centre of each tank so that it hung roughly two fish body lengths from the front and the back of the tank, and 140mm above the bottom of the tank. Behaviour was not analysed between ten and eleven minutes, in order to allow time for the novel object to stop moving, so that it did not interfere with EthoVision's tracking of the fish. Eleven minutes marked the beginning of the novel object test, which assessed individual boldness. There was a circular zone drawn in EthoVision which was roughly two body-lengths around the rubber bung (Fig. 2.2.8). A sign of a bold fish was that it spent a relatively large duration of time in this novel object zone, and there was a short latency to approach and a high frequency of visits to the novel object zone. By contrast, a sign of a shy fish was when a fish

did not approach the novel object at all. Anxiety levels are usually tested in a novel arena test over the first few minutes in a tank (Levin et al. 2007; Egan et al. 2009; Kalueff et al. 2013), but presumably, if the novel object was viewed as a potential threat, its presence would make the fish anxious. Thus, anxiety was again tested as proportion of time in the bottom zone.



*Figure 2.2.8:* Set-up for testing boldness, using a novel object (rubber bung). The amount of time the fish spends in the zone around the bung or in the bottom of the tank is recorded by EthoVision (tracks from the last 60 seconds shown in red). The fish on the left and in the centre are bold, while the fish on the right is not.

### ***Faulty tracking***

In some trials, EthoVision failed to track a fish for an extended amount of time, or tracked the novel object instead of the fish. I used the “subject not found” value, which gives the percentage of the trial in which EthoVision could not track the fish, to determine whether or not to edit the trial. I would edit tracks only when this number was higher than 10%. When the fish was frozen and not being tracked, I used the “track changes” feature of EthoVision to

artificially keep the tracked point in place until the fish moved again, thus allowing EthoVision to analyse the fish's position as normal. This was done for 19 individuals in total over all five time points. When a fish was moving around but not being tracked, I adjusted the detection settings and reanalysed the video in full. This was done for 12 trials in total over the five time points.

### ***Sociability test***

Sociability was tested separately, following the novel arena and novel object sequence. For the sociability test, I tested the males and females from each home tank as two separate groups. This meant that there were usually six fish per sociability trial, but as few as four in one male trial and one female trial at the week 27 repeat, because several fish had died prior to testing. The fish were tested in a white bowl, with a diameter of 244mm, filled with water to a depth of ~60mm (Fig. 2.2.9). After all the fish of one sex from a tank had been tested in the novel arena and novel object assays (2 trials), I placed them into the bowl and filmed their behaviour from above for ten minutes using a GoPro Hero 3+ camera on a stand (~430mm above the base of the bowl). The videos from the sociability test were analysed using idTracker (Perez-Escudero et al. 2014), a behavioural analysis program which can track the position of multiple individuals during a trial. idTracker output gives the position of each fish in pixels at each frame of the video. The resolution of the GoPro videos meant that VIE tags were not visible, so individual fish were not identifiable.



*Figure 2.2.9:* The sociability test set-up. Fish were placed into the bowl and filmed from above using a GoPro camera on a stand. VIE tags are not visible enough to individually identify fish.

### **2.2.3 Statistical analysis**

All data were analysed using R version 3.2.4 (R Core Development Team 2016). To simplify interpretation of the findings, only select response variables for each personality trait were chosen for analysis. Repeatability estimates were obtained for activity, exploration, and boldness traits using the package “rptR” (Stoffel et al. 2017), all with a Gaussian distribution except for the novel object approach, for which a binary distribution was used. The percentage of time spent immobile and percent of time in the bottom during the novel arena test were log-transformed. The statistical significance of repeatability estimates was inferred from whether or not confidence intervals included 0. Sex differences in continuous variables were analysed with linear mixed effects models in R using the package “lme4” (Bates et al. 2015), with sex as a fixed effect, and individual identity and time point included as random

effects. Cohen's d standardised differences were calculated to determine the effect sizes of the sex differences (Cohen 1992).

### *Activity*

Using data from the novel arena test, total distance travelled, mean velocity, and proportion of time spent immobile were identified as important markers of activity levels, but, because velocity and distance were so strongly correlated (Appendix 1), there were no meaningful differences between these two behaviours in analyses and figures. Distance travelled and the percent of time spent immobile were thus chosen as the proxy values for activity, but velocity data are shown in Appendix 1.

### *Exploration*

Using data from the novel arena test, exploration was calculated by using the standard deviation of the amount of time each fish spent in each of the nine zones. If a fish was exploratory and spent time in all of the zones equally, its standard deviation was low, whereas if a fish was not exploratory and spent most of its time in a few zones, its standard deviation was high. A demonstration of this measure is provided in Appendix 3.

### *Boldness*

Using data from the novel object test, interaction with the novel object was the main trait used to estimate boldness. The frequency of approaching the novel object was very low for most fish, and very high in only a few trials; accordingly, whether or not a fish approached the novel object was coded as binary (did approach *versus* did not approach). There were so many trials in which individuals never entered the novel object zone, that the actual values

for frequency of visits were no more informative than whether the fish ever approached the object (Appendix 4).

Finally, to check that the addition of the novel object into the tank had any effect on fish behaviour, GLMs (generalized linear models) were performed, comparing the distance travelled and percent of time spent in the bottom part of the tank in the novel arena and novel object trials.

### *Sociability*

The idTracker output gives the position of each individual at each frame of the video, as well as a probability estimate for how likely each value is to be correct. To analyse sociability, the idTracker output file from each video was run through a code in R (developed in my lab; Appendix 5) which removed all values for which the confidence was below 0.5. The code then gave the smallest distance from the nearest neighbour (NND) and the mean distance from all other fish (group cohesion) for each individual during the whole trial. The distributions for both measures were normal. Individual repeatability measures of sociability could not be obtained because of the low resolution of the videos, so the repeatability of tanks was calculated from the group means using the “rptR” package in R, with time point and tank number as random factors. There were four tanks, and males and females were tested separately, giving a sample size of eight groups for the repeatability model, each with five repeats. Sex differences were analysed as for the other personality measures, using a linear mixed effects model with sex as a fixed effect and tank number and time point as random factors.

### *Behavioural syndromes*

Comparisons were made between assays except for those between activity and anxiety, and exploration, because the findings for these behaviours came from two different methods for analysing the same footage. The behavioural syndrome analysis comprised each of the two measures for both boldness and activity, anxiety in the novel arena test, and the one measure for exploration, giving eleven total comparisons. To determine whether there were correlations between continuous variables, linear mixed effects models from the R package “lme4” were used, with time point and individual identity as random effects. Where the binary measure of novel object approach was being analysed, a GLM was used to check for associations between behaviours.

### *Deaths and exclusions*

Three females died of unknown causes after the initial testing, and three females and one male died after week 14, following a mating attempt in breeding boxes. Where fish had been excluded from later tests, their data were also removed from the earlier time points to avoid artificially inflating the repeatability measures with uneven sample sizes. There was one trial in the week 14 tests where one of the fish was lost by EthoVision in the boldness test, so it needed to be reanalysed. However, the video was corrupt, and so it could not be reanalysed. The data from that fish had to be excluded from all time points while I was analysing the boldness data. Thus, while my initial sample size was 48, by the time I was analysing the week 27 and overall data, the sample size for activity behaviours was 41 (20 females and 21 males) and, for boldness behaviours, it was 40 (19 females and 21 males). Exploration was reanalysed after the actual testing, so no information could be obtained from corrupt videos, and the sample size was 36 (15 females and 21 males).

## 2.3 Results

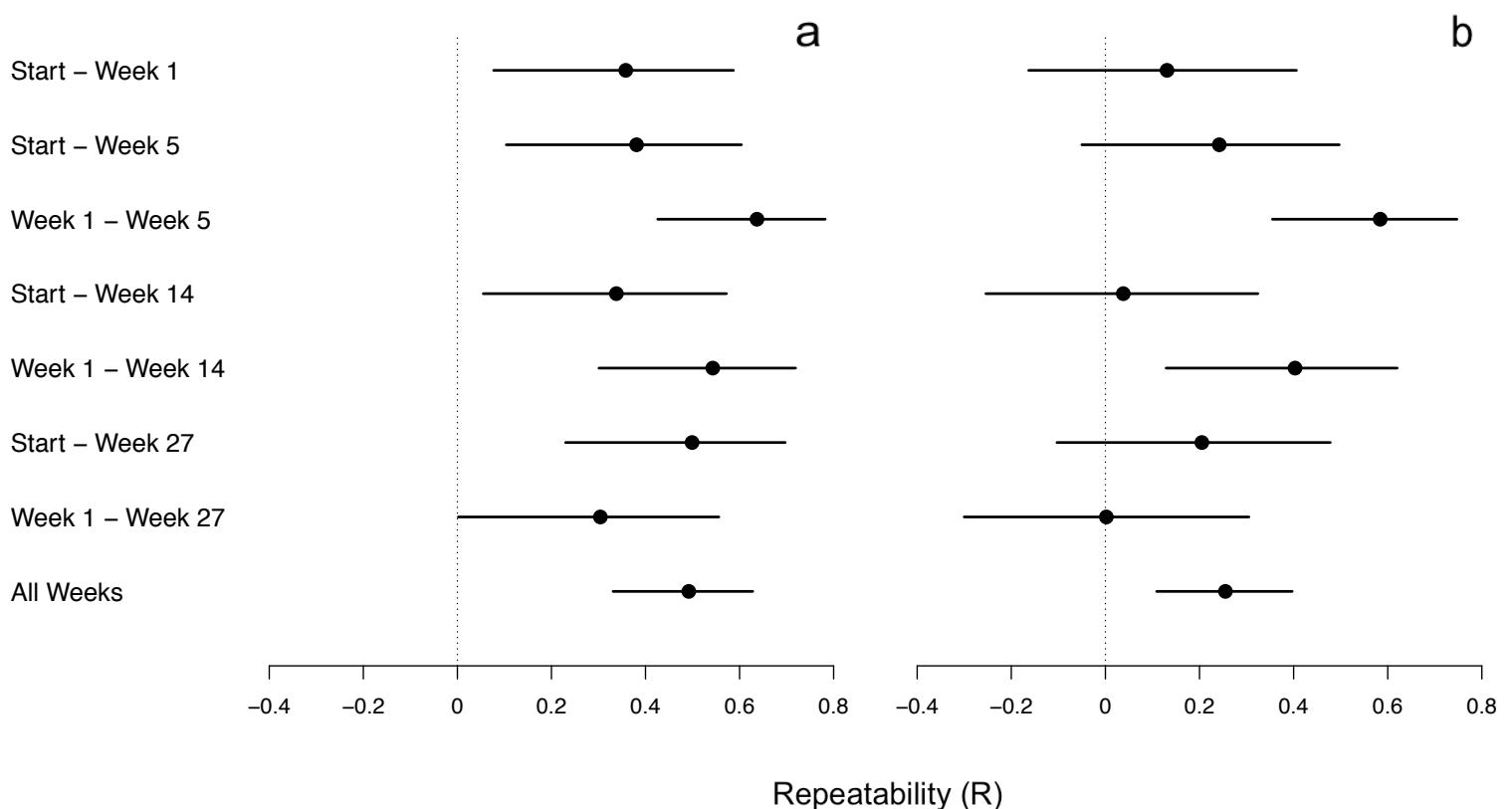
### 2.3.1 Activity

The total distance travelled ranged from 1020 to 5600 cm across all five time points (with mean 2880 cm and standard error (SE) 63.2 cm), while mean velocity ranged from 1.7 cm/s to 9.4 cm/s (mean 4.9 cm/s, SE 0.11 cm/s). The range of values for percent of time spent immobile was 2.9% to 62.1% (mean 18.1%, SE 0.9%). Anxiety, measured as percent of time spent at the bottom, ranged from 1.1% to 100% (mean 64.3%, SE 1.6%). Over the entire study period, including each time point, activity measured as distance travelled during a trial and the percent of time spent immobile was significantly repeatable (Fig. 2.3.1; for repeatability of velocity, see Appendix 1). Distance travelled ( $R = 0.49$ ; 95% CI = 0.35, 0.64) was overall more repeatable than immobility ( $R = 0.26$ ; 95% CI = 0.13, 0.42).

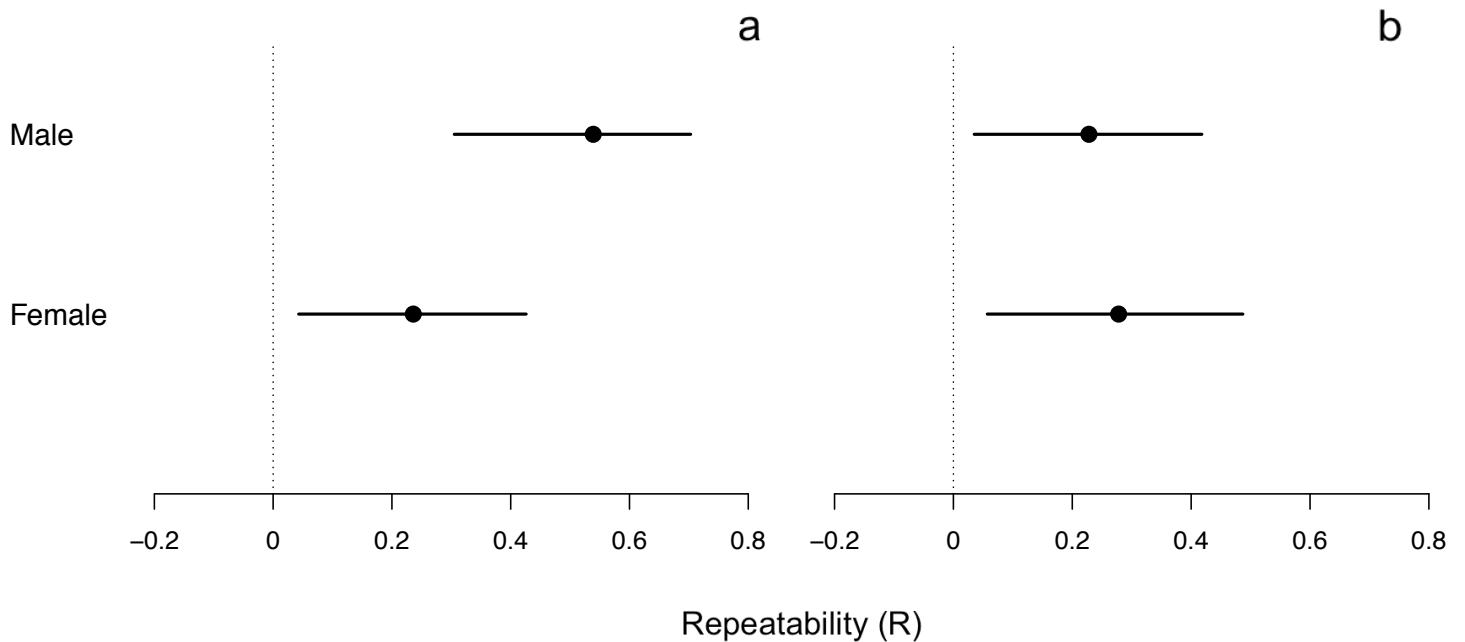
Pairwise analysis of each time point with the initial test and week 1 showed, with the exception of the week 27 repeat, that distance and immobility were both more repeatable from the one week repeat than from the initial trial (Fig. 2.3.1). The only significant difference in distance travelled was between the start and week 14, where fish in week 14 travelled an estimated 390cm farther than they did in the initial trial (standard error = 199, 95% CI = 2, 782); however, this difference was only marginally significant (Appendix 2 presents plots showing mean and individual changes in distance travelled over time).

It appears that distance travelled was more repeatable in males ( $R = 0.54$ ; 95% CI = 0.31, 0.70) than in females ( $R = 0.24$ ; 95% CI = 0.04, 0.43), but in percent of time spent immobile there was no major difference in repeatability between males ( $R = 0.23$ ; 95% CI = 0.04, 0.42) and females ( $R = 0.28$ ; 95% CI = 0.06, 0.49; Fig. 2.3.2). Males travelled (on average) 534cm farther in the activity assay than females did (standard error = 204; 95% CI = 134, 933cm).

There was more variation in the model between fish ( $SD = 586$ ) than between time points ( $SD = 114$ ). The effect size of the difference in total distance travelled between males and females was 0.62 (95% CI = 0.34, 0.90), which is both high and statistically significant (Cohen 1992). Although it appears that males spent on average less time immobile during the novel arena test, there was no significant sex difference (-1.9%, 95% CI = -4.24, 0.45%). There was, however, a small, significant effect size of 0.31 (95% CI = 0.04, 0.59).



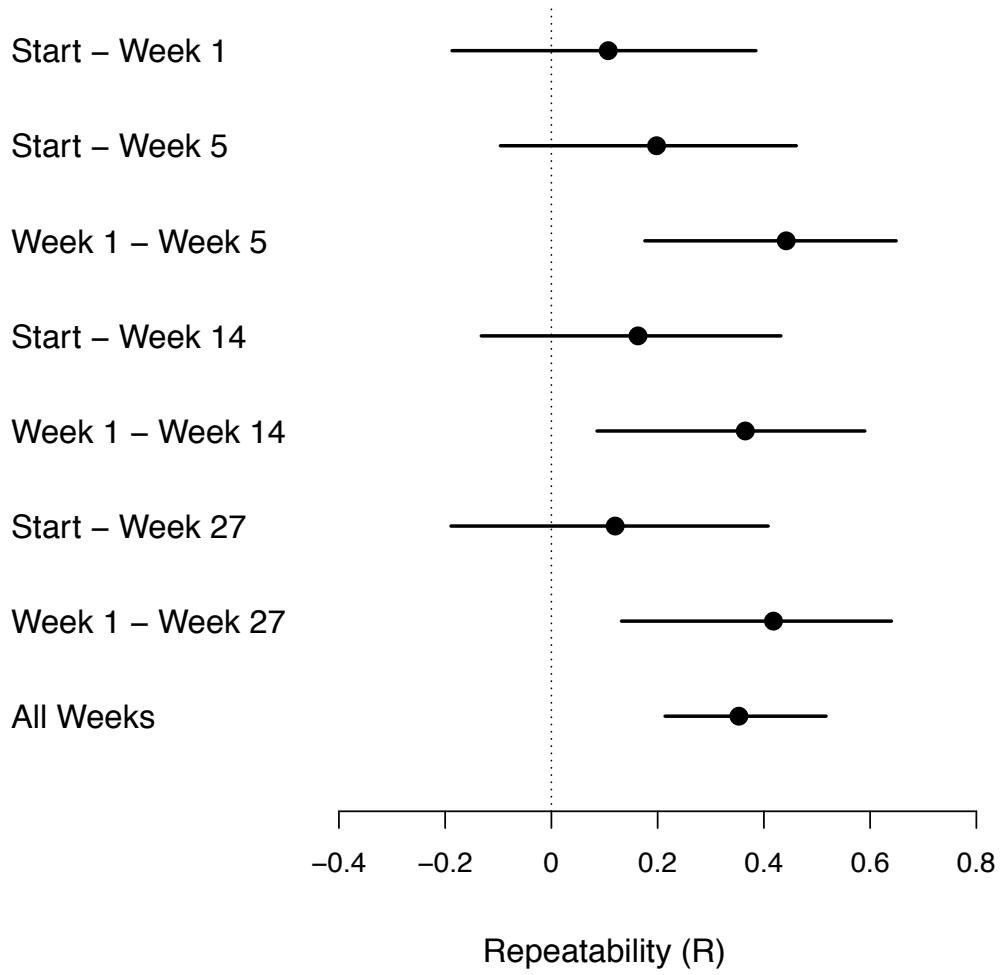
*Figure 2.3.1:* Repeatability estimates and 95% confidence intervals for distance travelled (a), and percent of time spent freezing (b) at each time point compared to the initial test and to the week 1 test, and for all weeks combined.  $n_{\text{fish}} = 41$ ,  $n_{\text{obs}} = 5$ .



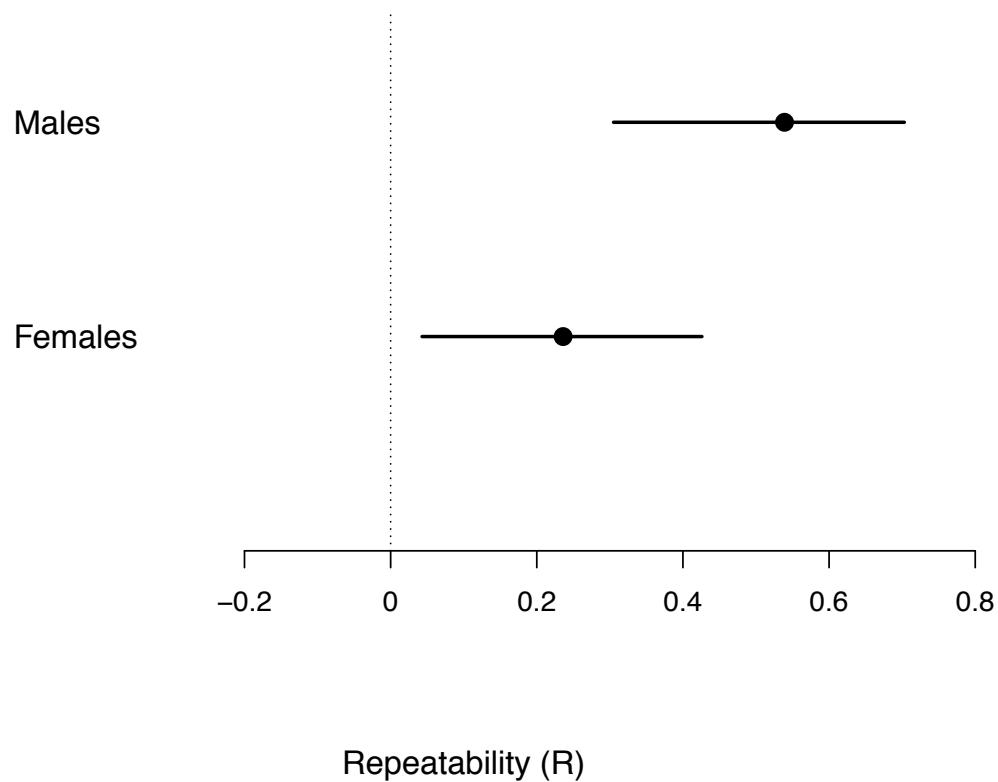
*Figure 2.3.2:* Repeatability estimates and 95% confidence intervals over all time points for males and females. Distance travelled is shown in *a*, while percent of time spent freezing is shown in *b*.  $n_{\text{males}} = 21$ ,  $n_{\text{females}} = 20$ ,  $n_{\text{obs}} = 5$ .

Anxiety was also significantly repeatable overall ( $R = 0.35$ ; 95% CI = 0.21, 0.52; Fig. 2.3.3).

As with distance, anxiety was more repeatable in males ( $R = 0.54$ ; 95% CI = 0.30, 0.71) than in females ( $R = 0.15$ ; 95% CI = -0.003, 0.39; Fig. 2.3.4). However, there was no significant difference in anxiety between males and females (-0.02; 95% CI = -0.21, 0.24), and the effect size for this was low and non-significant (0.06; 95% CI = -0.55, 0.67).



*Figure 2.3.3: Repeatability estimates and 95% confidence intervals for anxiety (measured as the percent of time spent in the bottom of the tank) at each time point compared to the initial test and to the week 1 test, and for all weeks combined.  $n_{\text{fish}} = 41$ ,  $n_{\text{obs}} = 5$ .*

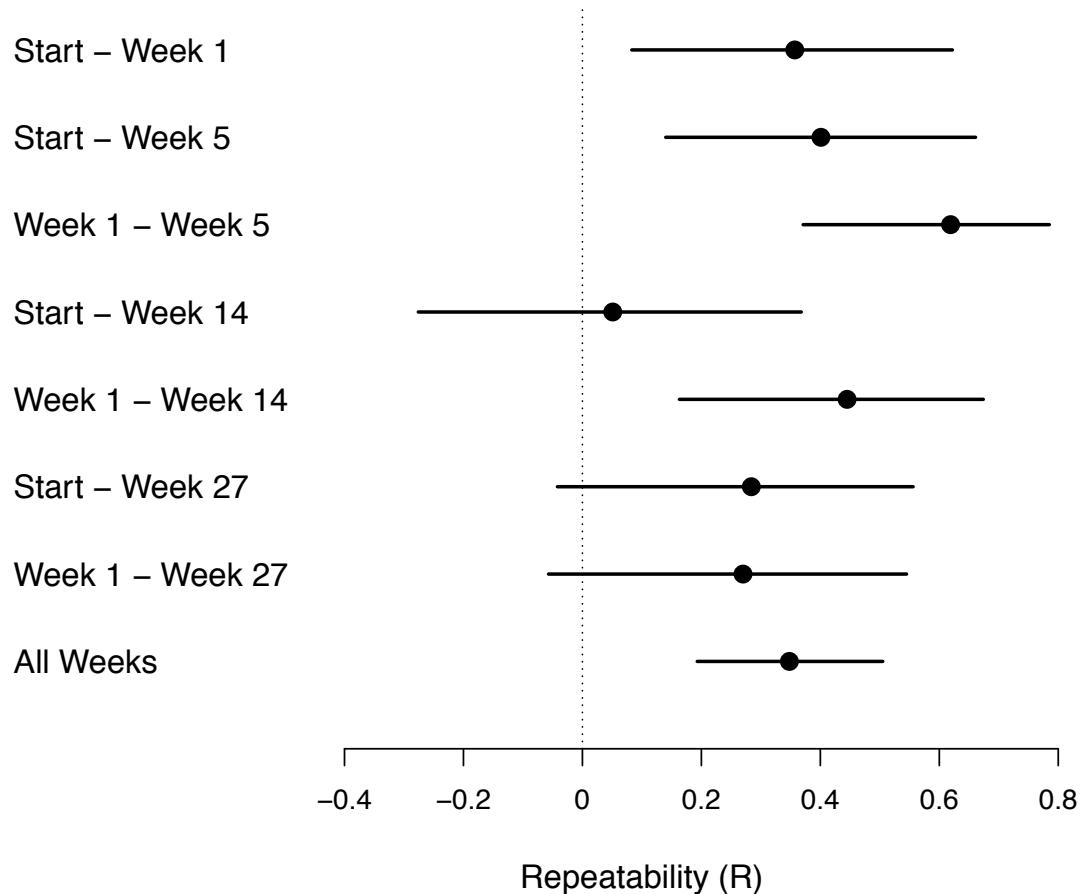


*Figure 2.3.4:* Repeatability estimates and 95% confidence intervals over all time points for anxiety (measured as percent of time spent in the bottom of the tank) for males and females.  $n_{\text{males}} = 21$ ,  $n_{\text{females}} = 20$ ,  $n_{\text{obs}} = 5$ .

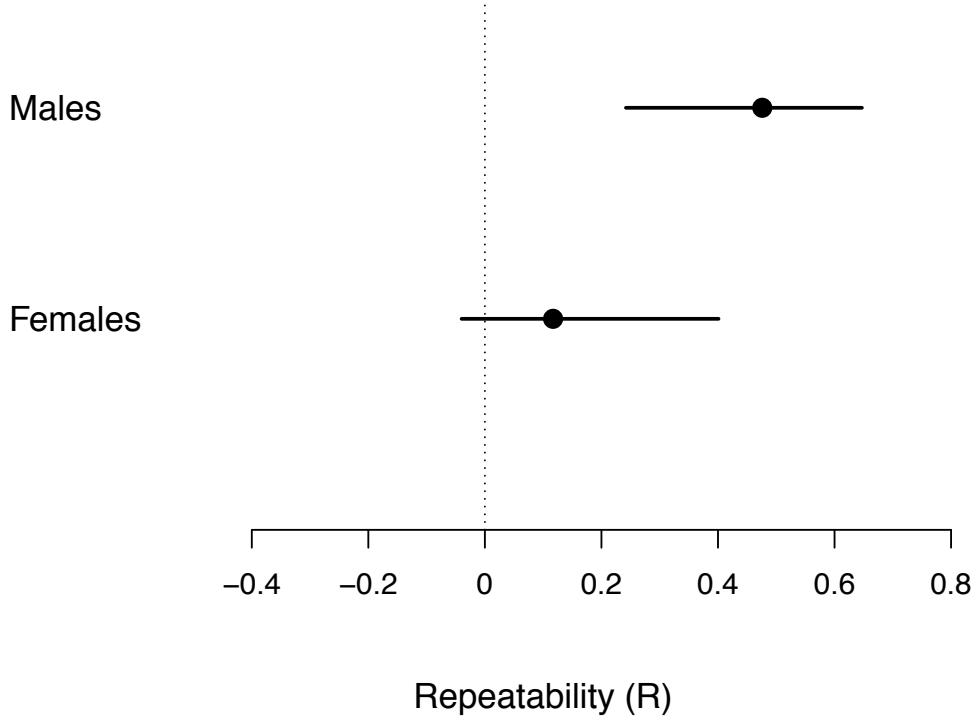
### 2.3.3 Exploration

The values for exploration—measured as the standard deviation of an individual’s duration in each of the nine zones—ranged from 140s (which indicates low exploration) to 18s (which indicates a high level of exploration), with a mean value of 59.7 seconds. Overall, exploration was significantly repeatable (Fig. 2.3.5). Repeatability from the initial test and week 1 did not show the same pattern as for the activity variables, where repeatability was higher from week 1, but exploration was also less repeatable at 27 weeks.

Exploration was significantly repeatable in males, but it was not significantly repeatable in females (Fig. 2.3.6). The model estimate suggested that males were slightly less exploratory than females (-2.55), but there was no significant sex difference in exploration (CI= -14.3, 9.16). The effect size for this difference was 0.1 (95% CI = -0.20, 0.40), which is low and non-significant.



*Figure 2.3.5:* Repeatability estimates and 95% confidence intervals for exploration over all time points compared to the start week and Week 1, and including all time points.  $n_{fish} = 36$ ,  $n_{obs} = 5$ .



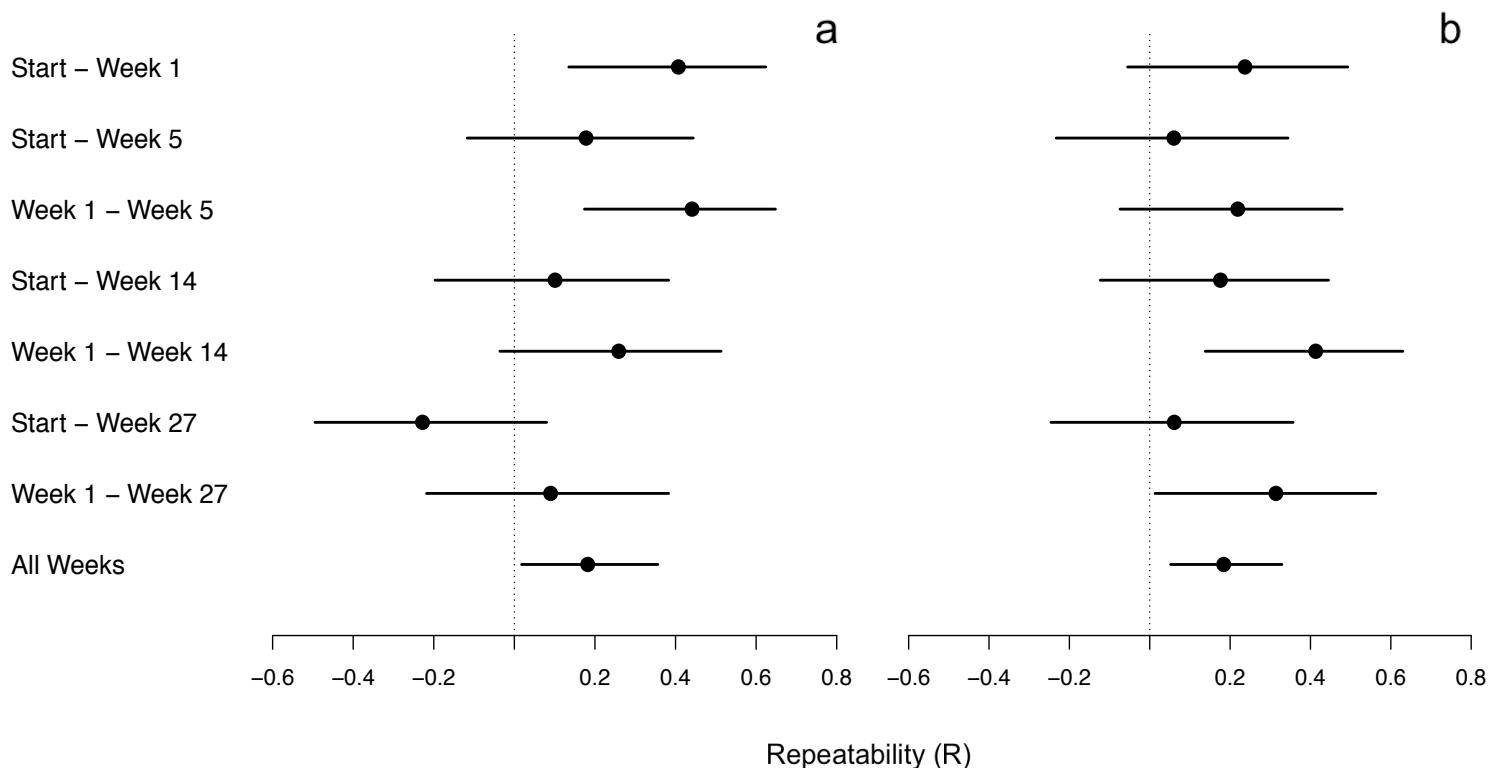
*Figure 2.3.6:* Repeatability estimates and 95% confidence intervals for exploration measured over 27 weeks.  $n_{\text{females}} = 15$ ,  $n_{\text{males}} = 21$ ;  $n_{\text{obs}} = 5$ .

### 2.3.2 Boldness

Most fish did not approach the novel object during the first three time points, and, even at weeks 14 and 27, only half of them entered the novel object zone (Appendix 4). The range for percent of time spent in the bottom during the novel object test was from 0.6% to 100%, with a mean of 77.1%.

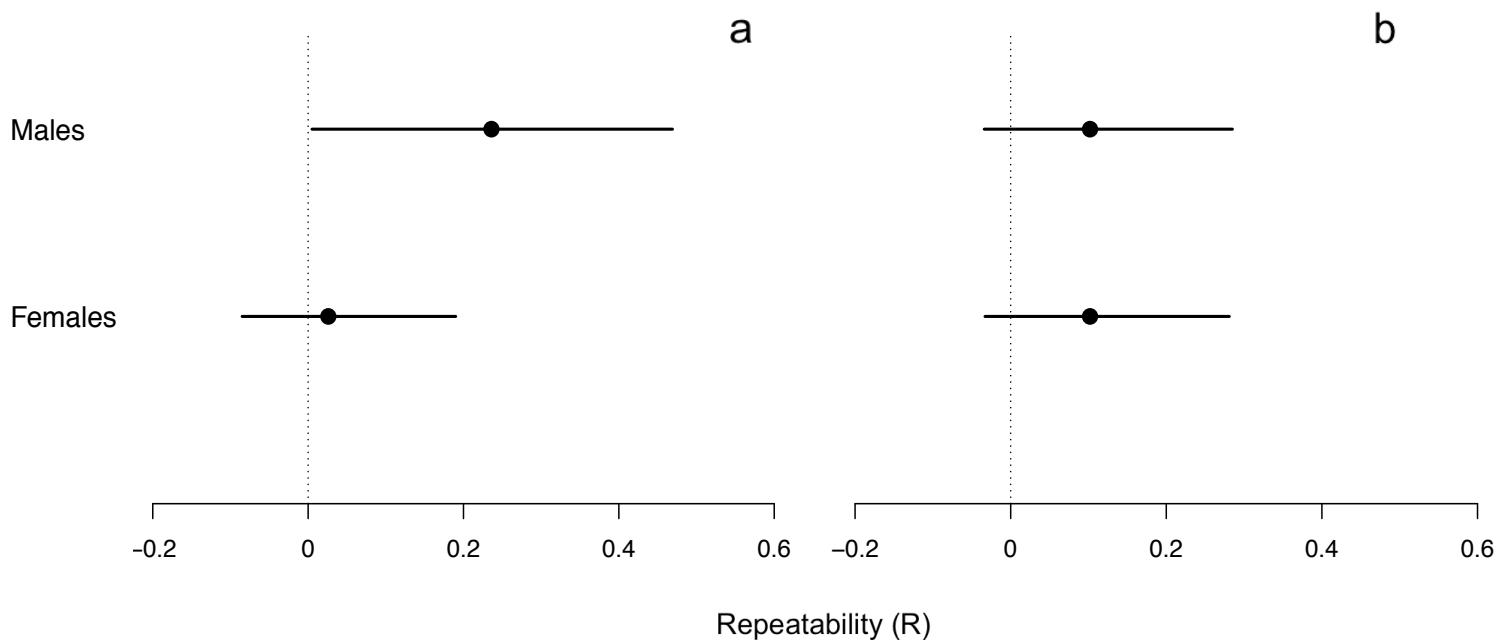
Whether or not a fish approached the novel object was repeatable overall, but with a much smaller estimate than the activity measures ( $R = 0.182$ ; 95% CI = 0.018, 0.356). Anxiety (the proportion of the trial in the bottom) was also repeatable overall, albeit with a similarly small estimate (0.18; 95% CI = 0.05, 0.33). When the repeatability estimates were compared pairwise from the start and week 1, the proportion of the trial in the bottom was largely not

repeatable except between week 1 and week 14 (Fig. 2.3.7b); this is in contrast with the novel arena trial, where time at the bottom of the tank was much more repeatable overall (0.35; 95% CI = 0.21, 0.52) and pairwise from the start and week 1 (Fig. 2.3.3).



*Figure 2.3.7:* Repeatability estimates and 95% confidence intervals for boldness over all time points from either the start week or Week 1, measured as whether the individual approached the novel object (a) and the proportion of time spent in the bottom part of the tank (b). Total combined repeatability over all weeks is also shown.  $n_{\text{fish}} = 40$ ,  $n_{\text{obs}} = 5$ .

Males were more repeatable in their approach to the novel object than were females (Fig. 2.3.8); the value for males was higher and just significant, but the confidence intervals were very wide. Approaching the novel object was not significantly repeatable in females. Males approached the novel object more than the females did (estimate = 0.189,  $p = 0.004$ ). The effect size of the difference was 0.413 (95% CI = 0.132, 0.693), which is low to moderate. There was no apparent sex difference in repeatability of time spent in the bottom (Fig. 2.3.8b). Males appeared to spend more time in the bottom, but the difference was marginally non-significant (estimate = 4.08%,  $p = 0.054$ ). The effect size of the sex difference was 0.276 (95% CI = -0.003, 0.555), which is low and non-significant.

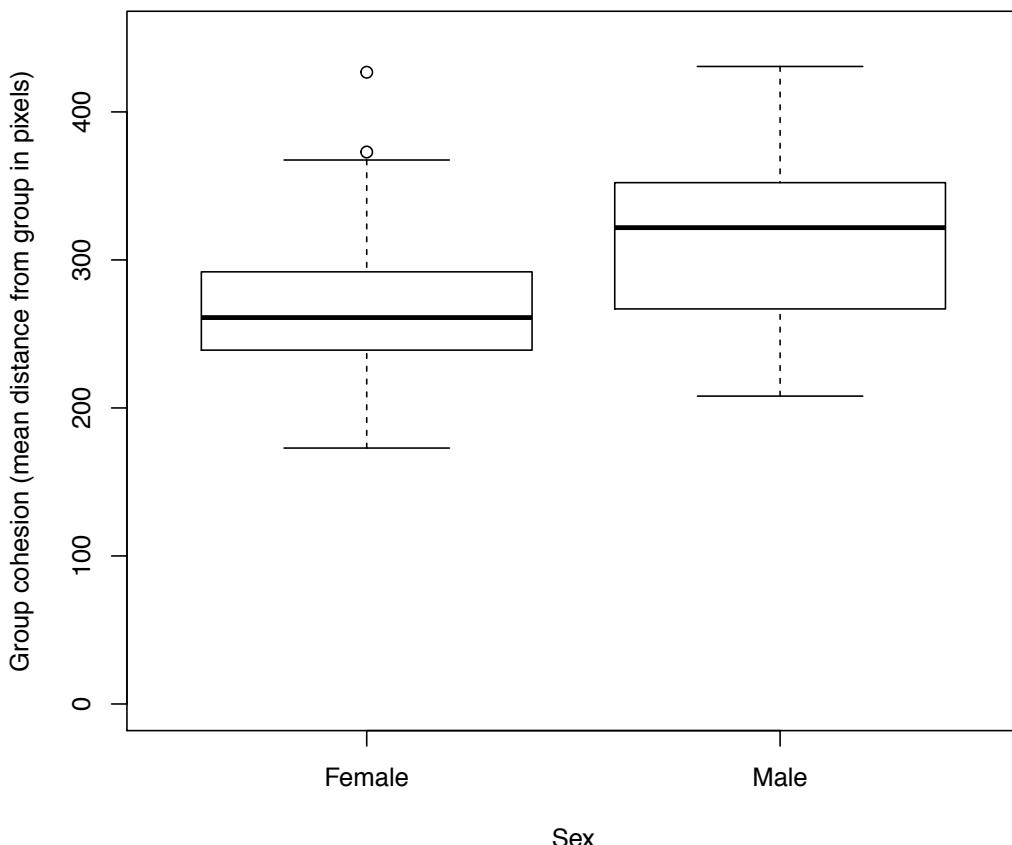


*Figure 2.3.8: Repeatability values and 95% confidence intervals for whether the novel object was approached (a) and proportion of the trial spent in the bottom part of the tank (b) by males and females, using data from all weeks combined.  $N_{\text{males}} = 21$  and  $n_{\text{females}} = 19$ .  $n_{\text{obs}} = 5$ .*

There was a significant difference between the novel arena and the novel object tests both in distance travelled and in amount of time spent in the bottom part of the tank; fish travelled (on average) 1360cm farther in the activity trial than in the boldness trial ( $t = 9.6$ ,  $p < 0.001$ ). The effect size was very large (1.54; 95% CI = 1.32, 1.76). The mean percent of time spent in the bottom part of the tank was higher in the boldness test (73.0%) than the activity test (64.1%;  $t = 4.56$ ,  $p < 0.001$ ). The effect size was moderate, at 0.33 (95% CI = 0.13, 0.52). Percent of time in the bottom was also less repeatable overall in the novel object test (0.18; 95% CI = 0.05, 0.33) than in the novel arena test (0.35; 95% CI = 0.21, 0.52).

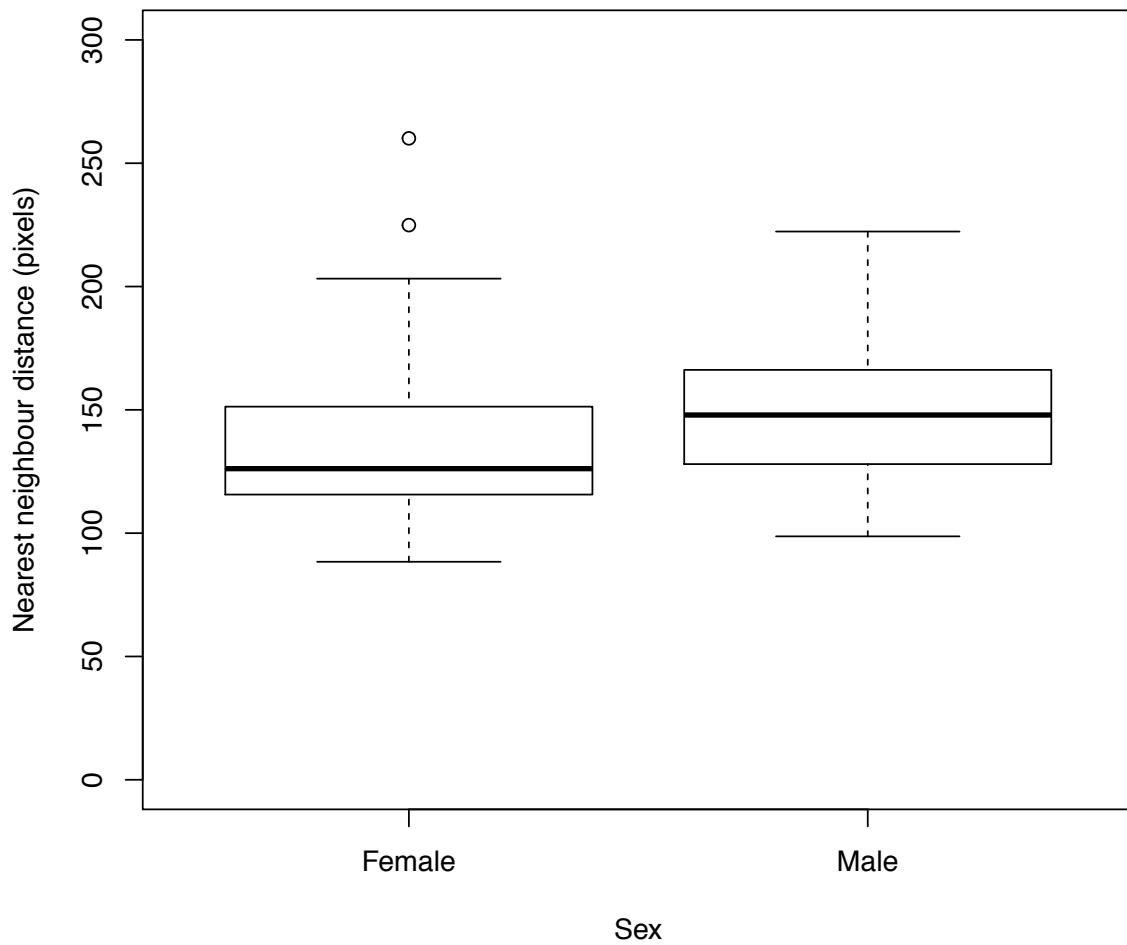
### ***2.3.4 Sociability***

Tracking success from the idTracker program was generally very good (Appendix 5), so few values had to be removed. At the tank level, group cohesion was significantly repeatable, but it had high variation ( $R = 0.48$ ,  $CI = 0.09, 0.71$ ). Males had, on average, significantly more space between individuals during the sociability tests (Fig. 2.3.9). The model estimated that males had 44.9 pixels greater distance between each individual and the other fish in the group ( $CI = 32.0, 57.9$ ). The effect size for sex differences in group cohesion was large at 0.9 ( $CI = 0.58, 1.23$ ).



*Figure 2.3.9:* Group cohesion (measured as mean distance in pixels from all other fish) for females and males during sociability tests over the five repeat trials, from the initial test to Week 27.  $n_{\text{tanks}} = 4$ ,  $n_{\text{obs}} = 5$  each for females and males. Boxplots include medians, upper and lower quartiles, and ranges.

The distance from the nearest neighbour (NND), however, was not significantly repeatable at the tank level ( $R = 0.12$ , 95% CI = -0.08, 0.55). There were also sex differences in NND (Fig. 2.3.10); males maintained significantly larger distances from the nearest neighbour than did females (estimate = 11.8 pixels, 95% CI = 4.29, 19.3). The effect size of the difference between males and females was moderate at 0.43 (95% CI = 0.15, 0.71).



*Figure 2.3.10:* Nearest neighbour distance (in pixels) for females and males over all of the five time points studied.  $n_{\text{tanks}} = 4$ ,  $n_{\text{obs}} = 5$  each for females and males. Boxplots include medians, upper and lower quartiles, and ranges.

### 2.3.5 Behavioural syndromes

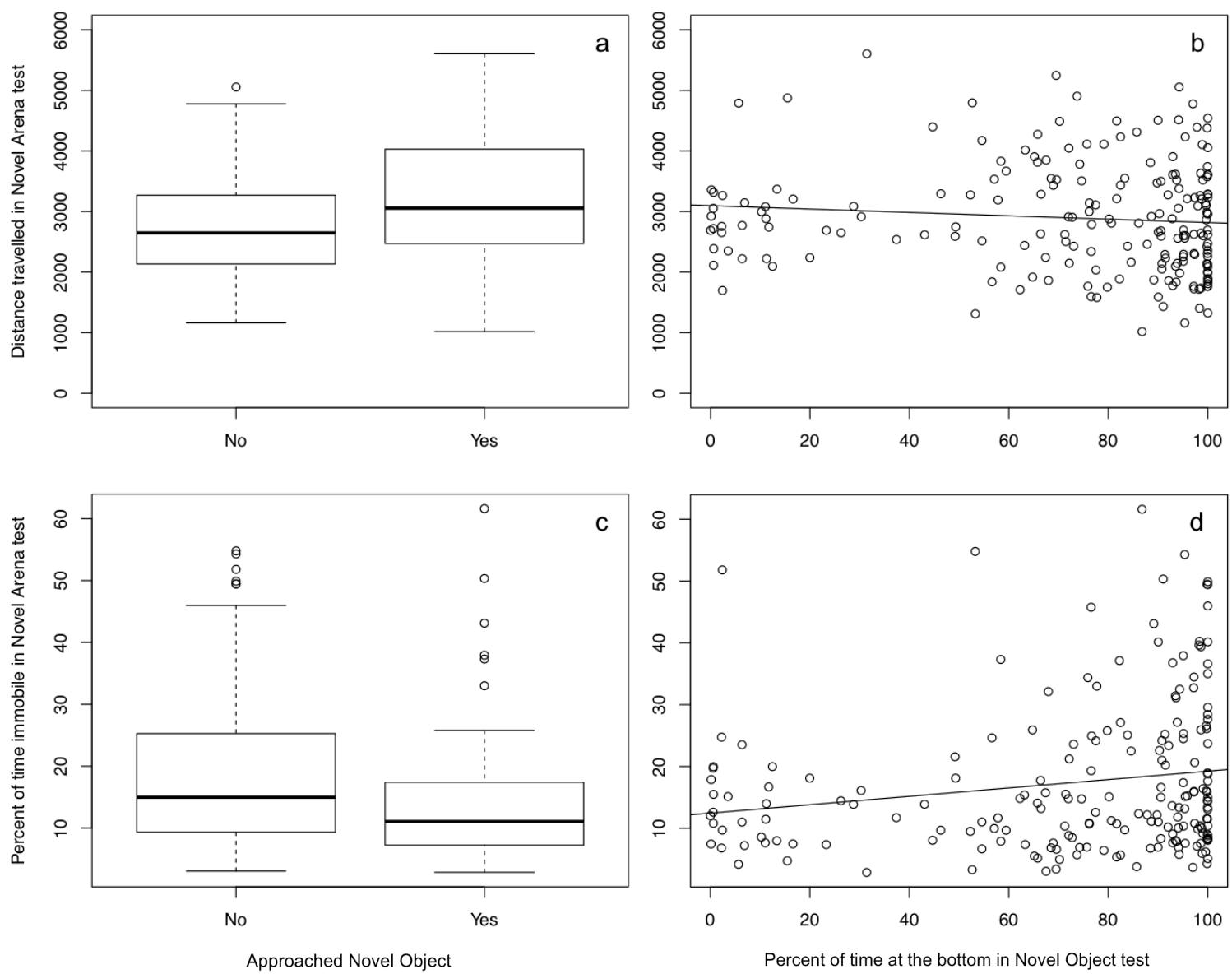
Distance travelled during the novel arena test was significantly associated with whether or not the individual approached the novel object; fish that did approach the novel object travelled (on average) 448cm farther than fish that did not ( $t = 3.38$ ,  $p < 0.001$ ; Fig. 2.3.11a). Distance travelled in the novel arena assay was not significantly associated with the percent of time spent in the bottom during the novel object assay (estimate -2.8, 95% CI = -6.04, 0.446; Fig. 2.3.11b). The percent of time immobile in the novel arena assay was significantly associated with both novel object approach (estimate -3.82%,  $p = 0.038$ ; Fig. 2.3.11c) and the

percent of time spent at the bottom (estimate 0.068%; 95% CI = 0.016, 0.12; Fig 2.3.11d).

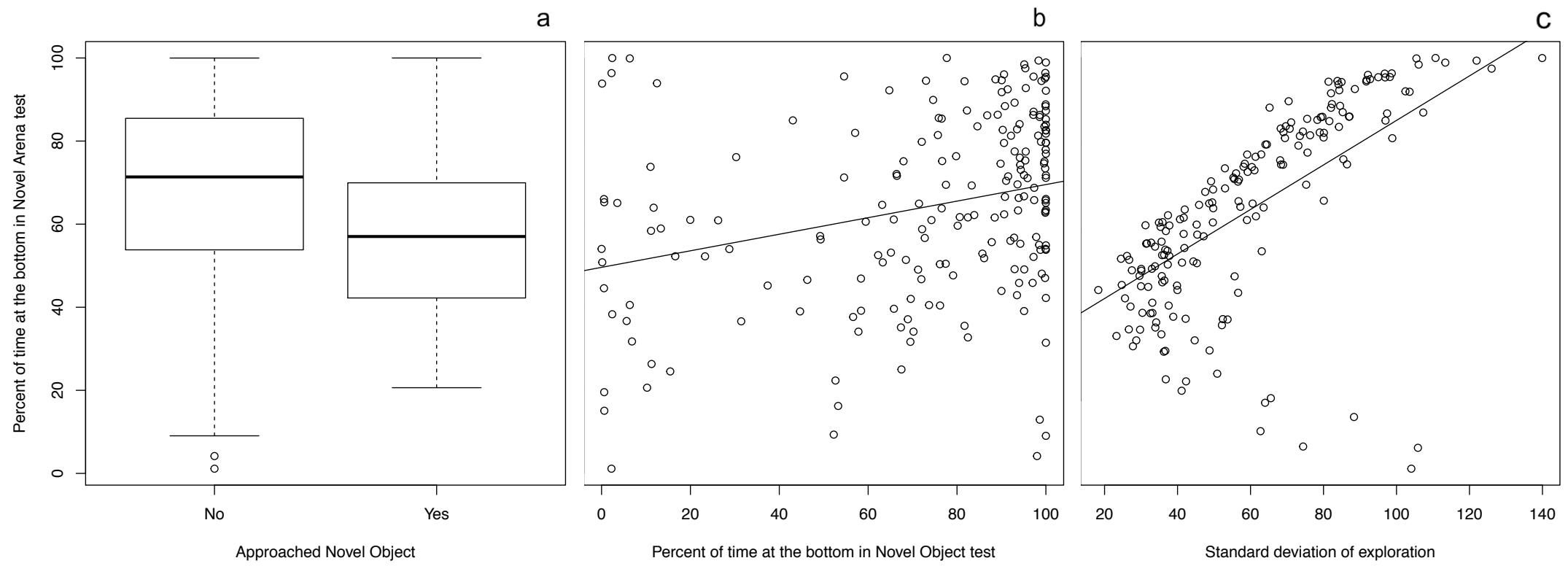
Fish that spent a large proportion of time immobile in the novel arena test tended to approach the novel object less, and to spend more time in the bottom during the novel object test.

There was a significant association between anxiety measured as percent of time at the bottom in the novel arena test and novel object approach; fish that approached the novel object spent on average 9.5% less time in the bottom during the novel arena test ( $t = -2.86$ ,  $p = 0.005$ ; Fig. 2.3.12a). Fish that were more anxious in the novel arena test were also more anxious in the novel object test; the correlation between these two behaviours was 0.64 (estimate 0.20, 95% CI = 0.12, 0.28; Fig. 2.3.12b). Exploration measured as standard deviation of frequency of entering each zone was significantly associated with anxiety in the novel arena test; more anxious fish tended to be less exploratory. The correlation was 0.68 (estimate 0.12, 95% CI = 0.02, 0.23; Fig. 2.3.12c).

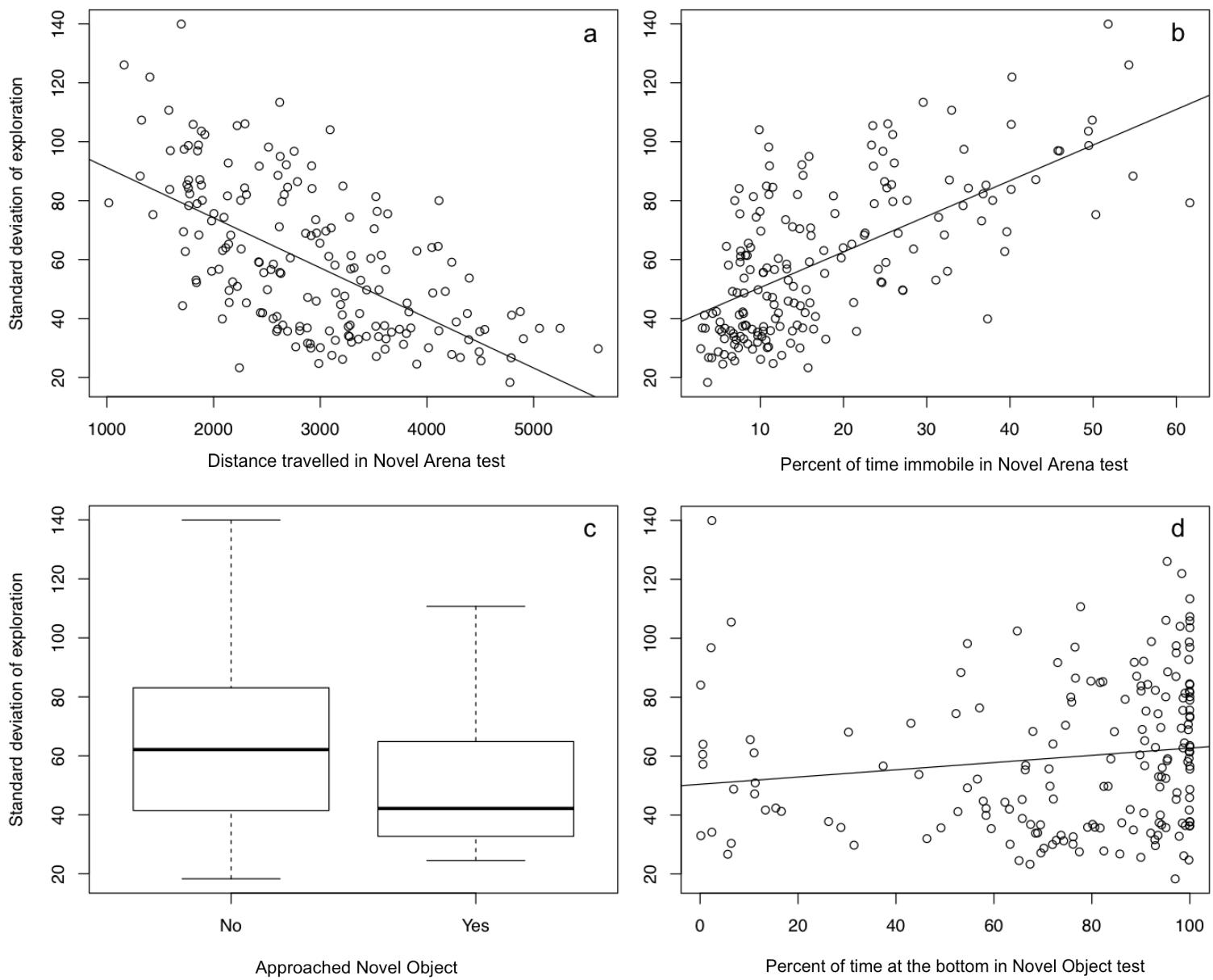
Exploration was also significantly associated with all of the values for both activity and distance. Fish that travelled farther were more exploratory, and the correlation was -0.87 (estimate -0.017, 95% CI = -0.02, -0.013; Fig. 2.3.13a). Fish that spent a larger percent of time immobile were less exploratory, and the correlation was 0.48 (estimate 1.21, 95% CI = 1.01, 1.41; Fig. 2.3.13b). Fish that did not approach the novel object tended to be less exploratory (estimate -14.2,  $p < 0.001$ ; Fig. 2.3.13c), and a larger percent of time at the bottom in the novel object test was associated with lower exploration, with a correlation of 0.68 (estimate 0.12, 95% CI = 0.02, 0.23; Fig. 2.3.13d).



*Figure 2.3.11:* Associations between the measures used to estimate activity and boldness; distance travelled in cm (a, b) and percent of time spent immobile (c, d) compared with whether the individual approached the novel object (a, c) and percent of time at the bottom (b, d).  $n_{\text{fish}} = 40$ , and  $n_{\text{obs}} = 5$  for each association. Boxplots are used to illustrate novel object associations, and scatter plots with regression lines for percent of time at the bottom.



*Figure 2.3.12: Associations between anxiety (measured as percent of time spent in the bottom of the tank) in the novel arena test and novel object approach (a), anxiety in the novel object test (b), and exploration measured as standard deviation of the cumulative duration spent in each zone (c).  $n_{fish}=36$ , and  $n_{obs}=5$  for each association.*



*Figure 2.3.13:* Associations between exploration measured as standard deviation of the cumulative duration spent in each zone and distance travelled in cm (a), percent of time spent immobile (b) during the activity test, whether the novel object was approached (c), and percent of time spent at the bottom (d) during the boldness test.  $n_{\text{fish}} = 36$ , and  $n_{\text{obs}} = 5$  for each association.

## 2.4 Discussion

The aim of this study was to investigate the repeatability of personality-related behaviours over 27 weeks, a longer period than is commonly tested, using zebrafish (*Danio rerio*) as a model. I predicted that behaviours related to activity and boldness would be repeatable, that there would be sex differences in these behaviours as well as in sociability, and that there would be correlations between personality traits; that is, that the fish would exhibit behavioural syndromes. Overall, individual activity and exploration, and sociability at the tank level were fairly repeatable over 27 weeks. Boldness was also repeatable, but to a lesser degree. There were also clear sex differences in aspects of activity, sociability, and boldness.

### 2.4.1 Activity

Both of the measures I examined in depth for activity (distance travelled and percent of time immobile) were significantly repeatable over the entire study period. Distance travelled was consistently more repeatable than proportion of time spent immobile, but the pattern across the time points is similar for both variables (Fig. 2.3.1). Repeatability from both the initial measurement and from the one week measurement was also compared for both behavioural measures and for anxiety. For all but week 27, repeatability estimates were higher and variation was smaller when comparing behaviour from week one than from the initial test for the activity measures, but not for anxiety. The pattern was very similar across the time points sampled in both distance travelled and proportion of time immobile, possibly because of the link between these two variables; that is, a fish which is immobile for most of a trial is likely to cover less distance than one which does not freeze at all. There is also a similar overall pattern for repeatability of anxiety over time. Higher repeatability from the one week test than from the start may have been because the fish needed some experience with the test tanks first before exhibiting their natural behaviour. Researchers have suggested that activity

should be measured in a non-novel environment to get an accurate measure of an individual's normal activity levels (Renner 1990; Réale et al. 2007). However, anxiety is usually tested in a novel tank (Egan et al. 2009; Parker et al. 2012). The exception to the pattern of greater repeatability in activity from week 1 than the initial assay was distance travelled in week 27; males appeared to travel less far in week 27 than in previous weeks (Appendix 2), but there was no change observed in the females. Anxiety, however, did remain repeatable between week 1 and week 27. The fish from this experiment were tested in a mirror aggression assay for a separate experiment twice in between the 14 week and the 27 week repeats, but the latter of these aggression tests was four weeks before the 27 week trial, and so I did not expect the other testing to affect the current experiment. It is unclear why the fish changed their behaviour, or why only the males were affected.

Males were more repeatable than females in the total distance travelled, and they also travelled farther on average, but there were no sex differences in either repeatability or actual values for the proportion of time spent immobile in the novel arena test (Fig. 2.3.2). Similarly, males were more repeatable in anxiety than females were (0.537 vs. 0.148; Fig. 2.3.2), but there was also no difference in the average amount of time spent in the bottom part of the tank. These findings that the behaviour of males was more repeatable than that of females are in contrast with a previous study on zebrafish, which found females to be more consistent in activity-related behaviour over a week than males (Tran and Gerlai 2013). In the Tran and Gerlai paper, however, fish were kept isolated for a week before testing, and were then tested every day for 7 days, and so there were methodological differences between that study and mine which could have contributed to the differences in findings.

## **2.4.2 Exploration**

Exploration—measured as the standard deviation of the cumulative duration spent in each segment of the superimposed grid during the novel arena test—was repeatable overall. The pattern of changes in repeatability values across the time points was similar to that of the activity measures, except that week 27 was repeatable neither from the initial test, nor from week 1 (Fig. 2.3.3). Similarities in patterns between activity and exploration measures are to be expected, because the estimates of these behaviours are derived from different measures of the same videos. On one hand, the non-repeatability of exploration at week 27 is different from activity, but the non-repeatable measures from week 27 did not prevent exploration from being repeatable overall. Exploration was more repeatable in males, but was not significantly repeatable in females; this is also similar to activity, but the difference between male and female repeatability is more extreme (Fig. 2.3.4). There was, however, no significant difference in the actual exploration values between males and females; thus, while the females were less consistent in their exploratory behaviour, there was no mean difference in the level of expression of the behaviour. There is abundant existing research into exploratory behaviour (e.g., Renner 1990; Dingemanse et al. 2002; Jones and Godin 2010; Carlson and Langkilde 2013; Thomas et al. 2016), and it is generally found to be repeatable. Of the sample of studies which examined the repeatability of exploratory behaviours in Table 1 (Chapter 1), the mean repeatability value was 0.4, which is similar to the overall value obtained in this study (0.348; although males were more repeatable, with 0.476), despite the majority of those previous studies testing repeatability over much shorter periods.

There is a suggestion that early life measures of behaviour may not reflect behaviours later in life (David et al. 2012; Herde and Eccard 2013). In voles, activity and exploration behaviour are repeatable over short periods but, across longer periods, behaviour is less consistent and

depends more on life stage (Herde and Eccard 2013). As mentioned in the introduction to this chapter, up to the age of ten months, male zebrafish are more active than females, but after 22 months of age, females are more active (Philpott et al. 2012). The zebrafish used in this experiment were tested from the age of ~five months over a fairly large portion of their lifespans, until the age of ~13 months; zebrafish in captivity typically live for over three years (Gerhard et al. 2002). Thus, while age-related effects could have been present in this experiment, the zebrafish were already of reproductive age at the start, and did not reach near their maximum lifespan or the age at which Philpott et al. (2012) observed altered effects of sex on behaviour, so any age-related effects were probably small.

#### **2.4.3 Boldness**

Both of the measures examined in the novel object test—novel object approach and percent of time at the bottom—were repeatable over 27 weeks, but these behaviours were less repeatable overall than activity. When looked at pairwise with the initial measure and week 1, the patterns of repeatability over time were again similar between the boldness and anxiety in the novel object test (Fig. 2.3.5) but were not similar to the patterns in the activity and exploration measures. However, novel object approach was repeatable only between the initial assay and week 1, and week 1 and week 5, while the proportion of time at the bottom was convincingly repeatable only between week 1 and week 14. There were no observable sex differences in these measures, and, when the repeatability estimates for males and females were calculated separately, neither were significantly repeatable (Fig. 2.3.6). Despite non-repeatability of some pairwise comparisons and of males and females separately, both of the behaviours measured in the novel object test remained repeatable overall, indicating some level of consistency in bold behaviour over time.

A common measure for anxiety in zebrafish is the novel tank diving test, where a fish that spends more time in the bottom part of a tank is considered more anxious (Levin et al. 2007; Parker et al. 2012; Collymore et al. 2015). The mean percent of time spent in the bottom part of the tank was significantly higher in the novel object test (73%) than in the novel arena test (64%), which indicates that the addition of the novel object did tend to make the fish more anxious. However, the lower repeatability in the novel object test ( $R = 0.182$ ) than in the novel arena test ( $R = 0.362$ ) may indicate that individuals were less consistent in their anxiety response in the second part of testing because of the presence of the novel object. Generally, the proportion of time in the bottom is measured over the first five or ten minutes in a novel tank; it may be also that the length of time for which the fish had already been in the tanks contributed to variation in behaviour. Nonetheless, the significant repeatability of proportion of the trial spent at the bottom of the tank indicates that anxiety is repeatable over time.

Overall, more appropriate methods for testing boldness may have led to better estimates of long-term repeatability in this experiment. The design of the test arena has previously been demonstrated to affect behavioural assay findings (Näslund et al. 2015). While the novel object test is a common method used to test boldness in zebrafish and in other taxa, it (of course) has limitations (Verbeek et al. 1994; Frost et al. 2007; Camín et al. 2016). In this experiment, so many fish never entered the novel object zone at all that the distribution of the data was heavily skewed, so interaction with the novel object was reduced to the binary outcome of whether or not the fish approached it, rather than analysing frequency or latency to interact. It may be that the novel object used, a rubber bung, was not relevant enough to the fish to elicit natural expression of bold or shy behaviours. Predator models or videos of predators have been used successfully in previous studies to examine boldness in fish; for example, a robotic predator in one study and an animated bird silhouette displayed on a

screen above a tank in another induced fear behaviour in zebrafish, (Luca and Gerlai 2012; Cianca et al. 2013). The repeatability of behaviour in these studies was not investigated, however, and so I cannot say that these methods would have been better. Nonetheless, with further testing, these more recently developed assays and the newer technology which they employ may transpire to be more appropriate tests of boldness than the traditional novel object test. In fact, Réale et al. (2007) suggested that a novel object test is more of an indication of exploratory behaviour than of boldness. On the other hand, Carter et al. (2012) endorsed its use over predator models in studies of boldness, and suggested that predator models might instead be testing anxiety. The length of time that the fish had already been in the test tanks at the start of the novel object assay may also have contributed to the low repeatability for both anxiety and for approaching the novel object; for example, the percent of time spent in the bottom in the boldness test was more repeatable over the first five minutes ( $R = 0.383$ ; 95% CI = 0.116, 0.604) than the last five minutes ( $R = 0.286$ ; 95% CI = 0.002, 0.526) between the initial test and Week 1, while it was not repeatable over the entire ten-minute test ( $R = 0.237$ ; 95% CI = -0.055, 0.493). A comparison of methods would be useful, to evaluate the pros and cons of both, and to determine whether objects which are more relevant to the test subjects induce more naturalistic displays of behaviour. There is also a need for further elucidation of what constitutes a measure of boldness as opposed to anxiety, in order for personality testing to more accurately categorise boldness behaviour.

Ideally, the videos from this experiment could be reanalysed to obtain the proportion of time spent immobile for the boldness test, as well as latency to leave the bottom zone of the tank. In tests using conspecific alarm cue, which is a chemical produced when a fish's skin is damaged and which alerts conspecifics in the area of nearby danger (Waldman 1982), freezing at the bottom of the tank is characteristic of alarm behaviour in shoaling fish (Egan

et al. 2009; Barbosa Júnior et al. 2012). The duration of an alarm response can be estimated by the amount of time it takes the fish to resume normal swimming behaviour in the water column (Egan et al. 2009); thus, the proportion of time spent immobile as measured in the novel arena test may have been more an indirect measure of boldness than of activity. Unfortunately, information on freezing and latency in leaving the bottom in the novel object test was not obtained at the time of recording the experiments, and time constraints have prevented further reanalysis. The addition of these two variables (along with a more appropriate novel object) would likely be a better test of boldness in future experiments.

#### **2.4.4 *Sociability***

Shoal cohesion was significantly repeatable at the tank level over 27 weeks, but the distance to the nearest neighbour was not. The wide confidence intervals from the repeatability model imply high variation, but this is not surprising considering that, with the males and females tested separately for each of the four tanks, the sample size in the model was just eight. As predicted, male fish tended to remain farther apart throughout trials than females did in both measures of sociability. Zebrafish have a natural tendency to shoal, so greater shoal cohesion may be a sign of anxiety (Stewart et al. 2012). Animals in a group may also take behavioural cues from their conspecifics, so, if one fish is particularly anxious, it could influence the others to behave in the same way (Ward 2012). The sociability tests were run after the novel arena and novel object tests. By this stage, each fish had been netted three times, and half of the fish in each sociability test run had been kept singly in 1 litre capacity breeding boxes while the rest of the fish in the trial were tested in the main assays. Stressful events can alter an animal's behaviour (Ferretti et al. 1995; Blanchard et al. 2001; Egan et al. 2009; Parker et al. 2012), so the fish may have behaved differently in the sociability trial than they usually would have done.

A further potential confounding factor is that not every tank had the same number of fish in it throughout the experiment; during the initial trials, each tank had six males and six females, but, from week 1, there were three tanks with only five females each. At the week 27 test, two tanks had just four males. This could have affected the repeatability of the data on the mean distance to the nearest neighbour; it has been demonstrated that zebrafish have a preferred stocking density which limits aggressive interactions and permits shoaling, and that their behaviour may change when this preference is not met (Pavlidis et al. 2013). There is evidence in mosquitofish (*Gambusia affinis*) that even asocial individuals prefer larger shoal sizes to smaller shoals when they choose to join a social group (Cote et al. 2012). If zebrafish show a similar preference, the small groups in which I tested sociability were probably not optimal. In a preference experiment, though, both longfin and wildtype zebrafish preferred a group of five conspecifics to isolation, but showed no discrimination between a group of five or 10 conspecifics (Kiesel et al. 2012). Regardless, it would be interesting to compare shoaling behaviour in small and large groups. Additionally, the design of the test arena has been shown to affect the results of behavioural assays (Näslund et al. 2015). A larger testing arena might have shown different findings, because the bowl in which the fish were tested did not allow for much space between individuals. Less sociable individuals could have elected to remain even further away from the group if it were possible. Additionally, the repeatability of sociability might also have been different when compared to the initial test or to week 1, as was seen for the other behaviours tested, but, because repeatability could be calculated only at the tank level for this behaviour, the sample size would be insufficient to enable a pairwise assessment of repeatability at different points throughout the experiment. Thus, I cannot say whether shoaling behaviour was less repeatable with the smaller shoals at week 27, or whether the presence of conspecifics might have relaxed the zebrafish sufficiently for the display of more normal behaviour from the beginning.

#### **2.4.5 Behavioural syndromes**

The findings from the behavioural association analysis largely did support the prediction that there would be associations between the different behavioural traits. The association between distance travelled during activity and the percent of time spent in the bottom during the novel object assay was the only non-significant finding from the behavioural associations analysis. Otherwise, fish that approached the novel object during the novel object test travelled farther and were more exploratory during the novel arena test. Anxiety in the novel arena test was associated with higher levels of boldness and anxiety in the novel object test. Fish that spent more time at the bottom of the tank in both the novel arena and the novel object tests tended to be less exploratory. The values for exploration were low if a fish was exploratory and high if a fish was not exploratory, so the negative relationship with distance travelled and the positive relationships with percent of time immobile and percent of time at the bottom also support the prediction that exploratory behaviour would be associated with activity and boldness.

It must be restated, however, that the activity, anxiety, and exploration measures come from the same test, using the same video footage, but with it analysed differently. Thus, caution must be used when drawing conclusions from these analyses because they do not constitute independent tests. The associations with boldness are mostly significant, but are also weak. This may have been because of the tests used to measure boldness; as discussed earlier, my tests may not have captured a true measure of the boldness of the test fish. In the literature, links between boldness, exploration, and activity are common and generally strong (Cote et al. 2010; Dziewczynski and Crovo 2011; Rudin et al. 2016). Thus, I can tentatively say that the zebrafish in my study probably were exhibiting a behavioural syndrome, despite the potential unsuitability of some of the tests used.

#### **2.4.6 Dissimilarities in estimates of repeatability**

Differences in repeatability estimates may be related to the assays used. For example, as discussed above, the proportion of time a fish spends in the bottom of the tank is generally accepted as a measure of anxiety. Furthermore, studies often use latency to emerge from a shelter as a measure of boldness (Wilson and Godin 2009; Harris et al. 2010; Hedrick and Kortet 2012; Mayer et al. 2016). It is possible, then, that activity, exploration, and boldness could be measured together in a single assay, thus eliminating the confounding factor of different amounts of time spent in the test tank. As long as caution was applied in comparing traits (to avoid pseudoreplication arising from traits being measured from the same video footage), this strategy of measuring multiple behaviours together might provide good estimates of behaviours, as well as saving time and effort on the part of the researchers.

There are factors other than the type of test used which can affect within-individual repeatability. Social behaviours in female primates change according to the current stage of the reproductive cycle (Rowell 1972), and female lizards are less active when gravid (Husak 2006). The origin of the population being studied is another factor which can affect repeatability of behaviours; wild-caught and captive-bred animals encounter different circumstances and challenges. There is greater variability in a wild environment to which individuals must adapt, and so it might be expected that certain behaviours would be less repeatable in wild populations (Dingemanse et al. 2002). Different populations of a species can also show different levels of repeatability in behaviours; diversity of perches visited was repeatable in just two populations of a rufous-collared sparrow (*Zonotrichia capensis*) in a study comparing three wild populations (van Dongen et al. 2010).

More relevant to this experiment is a finding that a predictable feeding schedule may promote exploratory behaviour in zebrafish; fish that were fed the same ration twice every day were more exploratory than fish given a randomly selected number of feeds (between 0 and 3) that differed each day, but with the same total amount of food (Holley et al. 2014). The zebrafish in my experiment had a regular schedule of three feeds daily, but timing did sometimes vary with feeder or between days, and especially with holidays, where fish are only fed two times per day and often not at the same time of day. The week 14 repeat test was done in mid-January, not long after regular feeding had resumed after the Christmas/New Year holiday. Week 14 was not repeatable for approach to the novel object, but it was for proportion of time at the bottom compared with Week 1. The changes to the feeding schedule could have contributed to variability in the behaviour of the fish. A further potential confounding factor is that up to 24 fish were tested in the same three tanks per day. Fish rely on chemical cues from conspecifics to assess the risk of predation (Brown et al. 2004). Thus, if there were particularly anxious fish being tested early in the day, the behaviour of fish tested later on might have changed to also be more anxious. Finally, netting is generally considered a stressful event for fish (Ramsay et al. 2009; Raoult et al. 2012; Pavlidis et al. 2013). I attempted to net each fish as quickly as possible to minimise disturbance, but some individuals were more difficult to net than others. Cortisol levels reach a peak 15 minutes after netting stress, and then decline until a return to baseline after 60 min (Ramsay et al. 2009). Differences in levels of netting stress may have thus reduced repeatability during testing, because some fish took longer to catch for testing, and so may have experienced more prolonged stress than others.

In essence, despite there being a seemingly endless list of factors which may influence behaviour even aside from potentially suboptimal testing methods, there were significant

estimates of repeatability in this experiment, which implies that, in zebrafish, personality-related traits are repeatable over time.

#### ***2.4.6 Conclusions***

The overall aims of this study were to determine whether personality-related behaviours are repeatable over time, whether there are sex differences in these traits or in the repeatability estimates, and whether any of these traits represent behavioural syndromes. I found that, despite some lower repeatability values for some behavioural measures (namely, novel object approach and percent of time in the bottom during the novel object test), overall, the behaviours studied were all significantly repeatable when tested at five time points over a 27 week period. Anxiety was more repeatable in the novel arena assay than the novel object assay, and anxiety levels were lower during the novel arena test. There were convincing sex differences in behaviours in distance travelled (that is, activity) during the novel arena assay, approach to the novel object (that is, boldness) in the novel object assay, and in mean nearest neighbour distance in the sociability test, but not in exploration or the percent of time immobile in the novel arena assay or the percent of time at the bottom of the tank in the novel object assay. Finally, there was evidence of an activity-boldness-exploration behavioural syndrome, but the conclusions drawn from this part of the analysis are more tentative because of non-independent data. It is important to study the repeatability of behaviours because it may provide insight into why and how personality is maintained in wild populations. Even studies carried out on captive populations and model organisms provide a base of knowledge which can be applied to both wild animals and to further behavioural testing in the laboratory. The findings from this study contribute to furthering our understanding of behavioural repeatability over time.

# **Chapter Three**

## **Changes in anxiety after a period of social isolation**

### **3.1 Introduction**

Personality involves behaviours that are generally consistent over time and across contexts (Réale et al. 2007). Despite this, some contexts may constitute enough of a challenge to an animal that its behaviour might change. Certain parasites can alter suites of linked behaviours within their host to facilitate transmission to the next host, in order to complete the parasitic lifecycle (Poulin 2013). An example of this is the freshwater amphipod *Gammarus pulex*, which usually group together as a form of predator defence. However, when infected by an acanthocephalan parasite (*Pomphorhynchus laevis*), for which *G. pulex* are an intermediate host, the amphipods do not show this aggregation response to a predator cue, and thus their susceptibility to predation is enhanced (Durieux et al. 2012). Another context in which behaviour may change is in pregnancy. Gravid female collared lizards (*Crotaphytus collaris*) have a slower maximum sprint speed, and are slower when foraging and escaping predators while gravid. Gravid females also remain nearer to refugia than non-gravid females (Husak 2006). As shown in Chapter 2, anxiety is repeatable over time in zebrafish. Accordingly, I will focus in this chapter on how the consistency of anxious behaviour might be altered by a change in context.

### ***3.1.1 Anxiety***

Anxiety is distinct from fear and panic. Fear is an emotion experienced as a response to an immediate, identifiable threat, while panic is fear experienced at its maximum intensity over a short period. Anxiety is the anticipatory fear of a potential and as yet unknown threat (Belzung and Philippot 2007; Maximino et al. 2012). The main distinguishing feature between fear and anxiety is the certainty or uncertainty of a threat (Belzung and Philippot 2007). The distinction generally made is whether it is state anxiety, which is acute and immediate, or trait anxiety, which is a chronic, individual tendency to react anxiously to a wide variety of events and challenges (Maximino et al. 2012). Human anxiety disorders are common; estimates of lifetime prevalence differ depending on the type of disorder, such as specific phobias (6-12%), social anxiety (about 10%), or generalised anxiety disorder (3-5%) (Kessler et al. 2010). In a study from the 1990s, the lifetime prevalence estimate of any anxiety disorder was 25% (Kessler et al. 1994). Thus, understanding the mechanisms and the outcomes of anxiety is important for human health.

There are aspects of human anxiety which are automatic responses essentially unchanged from far back within our evolutionary past, as well as elements which are instead linked to higher-level, more recently-evolved cognitive function (Belzung and Philippot 2007). Therefore, animals can be extremely useful in modelling some aspects of human anxiety. Many responses to threats or danger are conserved across several phyla; escape behaviour, reduction of movement, and reduction of non-defensive behaviours such as mating and feeding are seen in response to danger in animals ranging from molluscs and insects to fish and mammals (reviewed by Belzung and Philippot (2007)). Organisms ranging in level of complexity from protozoa to vertebrates display approach mechanisms to pleasant or attractive stimuli (to obtain food, mates, and shelter) and withdrawal mechanisms from

noxious or threatening stimuli (for defence, flight, or protection) (Schneirla 1959). These responses to unpleasant stimuli could be construed as simpler analogues of an anxiety response (Belzung and Philippot 2007). In recent years, fishes have been shown to have similar enough brain structure to humans that many tests of cognitive and social function can be generalised to humans and other vertebrates (Bshary et al. 2014). This includes tests of anxious behaviours.

There are ways of both attenuating and escalating an individual's level of anxiety in the short and long term. For example, ethanol has been shown to have anxiolytic effects in zebrafish (Egan et al. 2009; Parker et al. 2012), rats (Tornatzky and Miczek 1995), and humans (Kushner et al. 1996; Gilman et al. 2008). Reduction of anxious behaviours in response to anxiolytic pharmaceutical drugs has also been seen in multiple species of fish (Egan et al. 2009; Barbosa Júnior et al. 2012; Brodin et al. 2013). On the other hand, anxiety may be induced by exposing an animal to novelty; that is, introducing it to a new environment or object (Maximino et al. 2012). When an animal is allowed to habituate to this novel situation, however, the anxiety response is attenuated (Belzung and Philippot 2007). Another method is by using conspecific alarm cue, a chemical substance produced when mast cells in the fish's skin are damaged, which induces alarm behaviour in nearby conspecifics (Speedie and Gerlai 2008). Additionally, Egan et al. (2009) found caffeine to increase a range of anxious-type behaviours in zebrafish, including erratic movement, freezing, and reduced exploration. Clearly, there is a multitude of factors which can alter an individual's level of anxiety in a given situation. Furthermore, in animals which prefer to live in social groups, an important anxiety-inducing factor is sustained isolation from conspecifics (Egan et al. 2009; Parker et al. 2012; Pagnussat et al. 2013).

### ***3.1.2 Social isolation***

Stress is referred to as the processes that happen within the body while trying to maintain homeostasis in the face of a current or potential disruption (Yeh et al. 2013). In humans, there are strong, detrimental links between self-perceived social isolation and a range of physiological processes because of chronically higher stress levels (Cacioppo and Hawkley 2003; Teicher et al. 2006; Hawkley and Cacioppo 2010). More socially isolated individuals tend to have higher blood pressure than individuals who self-perceive as socially connected; this can lead to hypertension, which is a risk factor for a variety of diseases (Hawkley and Cacioppo 2010; Yang et al. 2016). The higher stress levels associated with social isolation can also contribute to slower wound healing and high rates of inflammatory disease (Cacioppo and Hawkley 2003; Cole et al. 2007). Similarly, the physiological markers of stress can be clearly seen in animals kept in isolation. Changes in cortisol levels are highly correlated with behavioural indices of anxiety in zebrafish (Egan et al. 2009) and in male rodents (Pagnussat et al. 2013). Isolation from conspecifics can elicit more aggressive behaviour in zebrafish after five days (Larson et al. 2006) and even overnight (Teles et al. 2013). This indicates that social animals and humans show similar effects of isolation from a social group.

Social isolation stress appears to have the greatest effect when it happens during early life; brain function and morphology can develop abnormally as a result, sometimes leading to psychological and personality dysfunction (Teicher et al. 2006; Steenbergen et al. 2011). It is hypothesised that the induction of stress responses during juvenile development may be the cause of isolation-related issues (Teicher et al. 2006). In rats, there is a period soon after birth where stressors have no adverse effect on the developing juvenile, presumably to protect the brain from the harmful effects of high levels of glucocorticoids (Sapolsky and Meaney 1986;

Teicher et al. 2006). However, the quality of maternal care received during this period can contribute to the development of individual variation in stress reactivity later on in life (Francis et al. 1999; Caldji et al. 2000). This is thought to be partially due to the epigenetic effects of maternal care. Differences in quality of maternal care alter DNA methylation and chromatin structure in the glucocorticoid receptor genes of offspring, and this causes changes in the way these offspring react to stressful events (Francis et al. 1999; Weaver et al. 2004). It seems that, rather than the events themselves, it is the individual's stress response to adverse events which leads to negative health and behavioural outcomes (Teicher et al., 2006).

Zebrafish are highly social (Miller and Gerlai 2007; Spence et al. 2008). They prefer to be housed in groups of 10 fish per 2L of available water volume (Pavlidis et al. 2013). Zebrafish that are housed individually for three weeks show higher levels of anxiety in novel tank diving and light-dark choice tests (Collymore et al. 2015). Zebrafish that are tested alone tend to be more stressed in the absence of their normal shoaling activity, and so their behaviour may be more variable (Pagnussat et al. 2013). Similarly, mosquitofish explore a novel environment more readily when in the presence of conspecifics than when alone (Ward 2012). In another study, however, zebrafish housed individually for 30 days showed lower cortisol responses to events which usually cause stress, such as netting for tank transfer and tapping on the tank, because of a lack of feedback they would usually have from conspecifics also responding to the same stressor (Giacomini et al. 2015). Social isolation in zebrafish can induce higher levels of aggression after periods as short as five days (Larson et al. 2006) or even overnight (Teles et al. 2013). Because the structure and function of the stress-regulating systems are similar in humans (the hypothalamic-pituitary-adrenal axis) and in zebrafish (the hypothalamic-pituitary-interrenal axis), findings from studies conducted on zebrafish are highly applicable to research on human stress (Steenbergen et al. 2011).

Anxiety in zebrafish can be tested in a number of ways, including light-dark tests and the novel environment diving response (reviewed by Maximino et al. (2012). The novel tank diving test uses the zebrafish's natural tendency to dive to the bottom of a novel environment to test stress and anxiety; it is inferred that the longer the fish spends in the bottom part of the tank instead of exploring the tank in the water column, the more stressed and anxious it is (Levin et al. 2007; Parker et al. 2012). Zebrafish also tend to either move faster and more erratically or to freeze when anxious (Parra et al. 2009; Kalueff et al. 2013). While the average adult zebrafish spends about 55% of the first five minutes in a novel tank swimming at the bottom (Levin et al. 2007), anxiolytic and anxiogenic substances can alter this tendency. Nicotine and anxiolytic drugs (such as diazepam or the chronic application of fluoxetine) can reduce the amount of time spent at the bottom of the tank (Levin et al. 2007; Bencan et al. 2009). There is also a reduction in diving response when the tank is not novel (Bencan et al. 2009). Anxiogenic compounds like caffeine and conspecific alarm pheromone increase the diving response (Wong et al. 2010). However, in Chapter 2 of this thesis, I demonstrated that the percent of time spent in the bottom of the tank during the first ten minutes was repeatable over time. Additionally, personality-related traits like anxiety are generally accepted to be repeatable across contexts (Cloninger 1986; Dall et al. 2004). It is possible, however, that a period of social isolation outside the norm for zebrafish may be enough of a challenge to disrupt the repeatability of anxiety.

### ***3.1.3 Aims and hypotheses***

Seeing as zebrafish are social animals that have been shown to prefer fairly high stocking densities, and anxiety may be induced when being held in conditions outside of the norm, it follows that a period of social isolation might lead to higher levels of anxiety than the same

period of time spent in a group, despite anxiety-related behaviour being generally repeatable within individuals from one trial to another. In doing this study, I aimed to investigate whether a period of social isolation would change a personality-related behaviour, and thus, whether isolation from conspecifics is outside the normal range of contexts under which behaviour is repeatable. I did this by phenotyping a cohort of zebrafish and separating them into anxious and non-anxious groups, and then exposing them to a period of social isolation vs. group living. I predicted that, after being housed in isolation, non-anxious fish would become more anxious. I also predicted that being housed in groups would have no effect on anxiety levels in initially anxious and non-anxious fish, because anxiety is repeatable. Housing conditions may also affect levels of activity, and activity is linked to anxiety (Egan et al. 2009; Gerlai 2013), so I expected to see that housing conditions outside the norm (that is, social isolation) might result in changes in the total distance travelled and mean velocity during a trial. Specifically, I predicted that distance and velocity would be higher in the isolated fish.

## **3.2 Materials and methods**

### ***3.2.1 Subjects***

The subjects of this study were male zebrafish (*D. rerio*) bred in the Otago Zebrafish Facility. The fish were the progeny of ~20 males and 20 females allowed to batch spawn on the 6<sup>th</sup> and 7<sup>th</sup> of September 2016. The fish were moved into holding tanks in the University of Otago Department of Zoology between mid-January and mid-February 2017. See Chapter 2 for holding tank and light cycle/temperature specifications, as well as feeding regimes.

### ***3.2.2 Experimental overview***

The amount of time spent in the bottom portion of a novel tank was used as a proxy measurement for anxiety. This measure was chosen because it has been used to estimate anxiety extensively in previous literature (Levin et al. 2007; Bencan et al. 2009; Egan et al. 2009; Parker et al. 2012; Kalueff et al. 2013), and because it was repeatable in the experiment in Chapter 2 of this thesis. In short, the current experiment involved phenotyping a cohort of fish to categorise them as “anxious”, “somewhat anxious”, “somewhat non-anxious”, and “non-anxious”. Fish were then kept for three weeks either isolated or in small groups, and then phenotyped again to see how each individual deviated from its previous classification.

### ***3.2.3 Experimental protocol***

#### ***Initial phenotyping***

A total of 145 fish were sorted into four categories based on the proportion of time spent in the bottom half of a novel arena during a ten minute trial. If a fish spent less than 40% of its time in the bottom of the tank, it was classified as non-anxious, whereas 40-55% indicated a fish that was somewhat non-anxious. If a fish spent 55-70% of its time in the bottom of the tank, it was classified as somewhat anxious, whereas more than 70% of the trial spent in the bottom half of the tank indicated an anxious fish. The justification for these splits came from the spread of the anxiety data from the five week part of my repeatability experiment, because anxiety was repeatable up to this time period (Chapter 2). The first group of fish were phenotyped on the 14<sup>th</sup> and 15<sup>th</sup> of February. There were not enough non-anxious fish after this, however, so more fish were phenotyped on the 6<sup>th</sup> of March. After both phenotyping sessions, there were roughly twice the number of anxious fish as any other group; 62 anxious, 36 somewhat anxious, 25 somewhat non-anxious, and 27 non-anxious fish.

The set-up for phenotyping anxiety and activity was the same as that for testing activity used in my repeatability chapter (Chapter 2). Each fish was placed into a tank (300mm H x 270mm W x 154mm D) which had been filled with half housing system water and half fresh sump water. The trials lasted ten minutes and I tested three fish at a time, one in each of the three tanks. I used EthoVision XT (Noldus) to track the proportion of time each fish spent in the bottom part of the tank (Fig. 3.2.1). Only male fish were used because I had previously found that males were more consistent in their anxiety behaviour than females (see Chapter 2). Twenty anxious and 20 non-anxious fish were randomly selected from the 62 anxious and 27 non-anxious fish for use in the social isolation experiment.



Figure 3.2.1: Arena settings used in EthoVision during phenotyping.

### **Social isolation**

Space constraints meant that only 20 tanks were available at a time, so the social isolation experiment was conducted over two trials. The outer sides of the holding tanks were covered in black plastic so that fish in adjacent tanks could not see each other. For each trial, the twenty tanks were separated into 5 anxious fish housed in groups, 5 anxious isolated fish, 5 non-anxious fish housed in groups, and 5 non-anxious isolated fish. Group tanks consisted of the focal individual plus four random mixed-sex fish that were identifiable by visual implanted elastomer tags from fish that were implanted for a different experiment (see Chapter 2 methods). Note that focal fish were not tagged due to time constraints on when this experiment could take place. Fish must be tagged at least 2 weeks prior to handling for experiments, and even longer time frames are required when moving fish from one facility to the other, as required for phenotyping in this study; hence, focal fish were not individually

identifiable. The first cohort were held in the system for three weeks from the 17<sup>th</sup> of March, and phenotyped on the 7<sup>th</sup> of April. The second cohort of fish were held for three weeks from the 13<sup>th</sup> of April and were phenotyped on the 4<sup>th</sup> of May. Fin clips and brains were collected from all 40 fish for future gene expression analyses (data not included in this thesis due to delays with quantitative PCR).

### ***3.2.4 Data analysis***

Statistical analysis was performed using R version 3.2.4 (R Core Development Team 2016). An ANOVA was run to confirm that the percent of time spent in the bottom was different between anxious and non-anxious groups at the initial testing. The distributions of values for distance travelled and mean velocity after the test period were normal. The influence of treatment (group or isolated) and pre-test anxiety (anxious or non-anxious) on anxiety (percent of time spent in the bottom of the tank), as well as velocity and distance travelled during the trial, were analysed using generalised linear models (GLMs). Because the experiment was run in two separate trials, trial was originally included as a factor, but showed no significant effects or interactions. Thus, trial was not included in the final models. The Cohen's d standardized difference (effect size) between before and after behaviours was calculated using pooled standard deviation (Cohen 1992; Wilson 2001).

### 3.3 Results

The difference in the percent of time spent in the bottom part of the tank between anxious and non-anxious fish before the experiment was significant (ANOVA;  $F_{1,88} = 601.8$ ,  $p < 0.001$ ; Fig. 3.3.1). The higher proportion of time spent in the bottom of the tank by anxious vs. non-anxious fish is clear when visualized in a heatmap (Fig. 3.3.2). However, anxious fish did not travel different distances (ANOVA;  $F_{1,87} = 0.033$ ,  $p = 0.86$ ; Fig. 3.3.3) or at different velocities (ANOVA;  $F_{1,87} = 0.479$ ,  $p = 0.49$ ; Fig. 3.3.4) from the non-anxious fish.

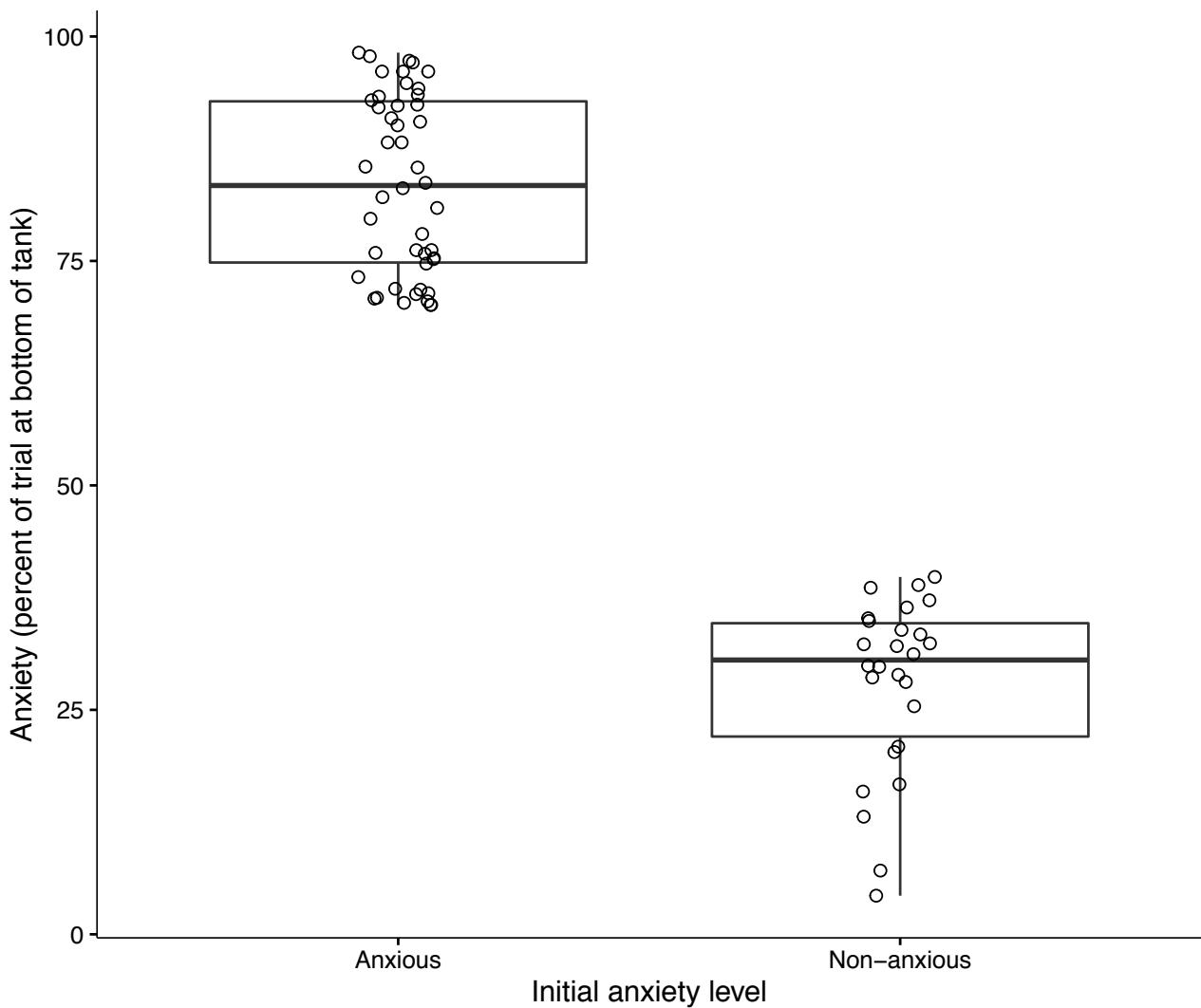
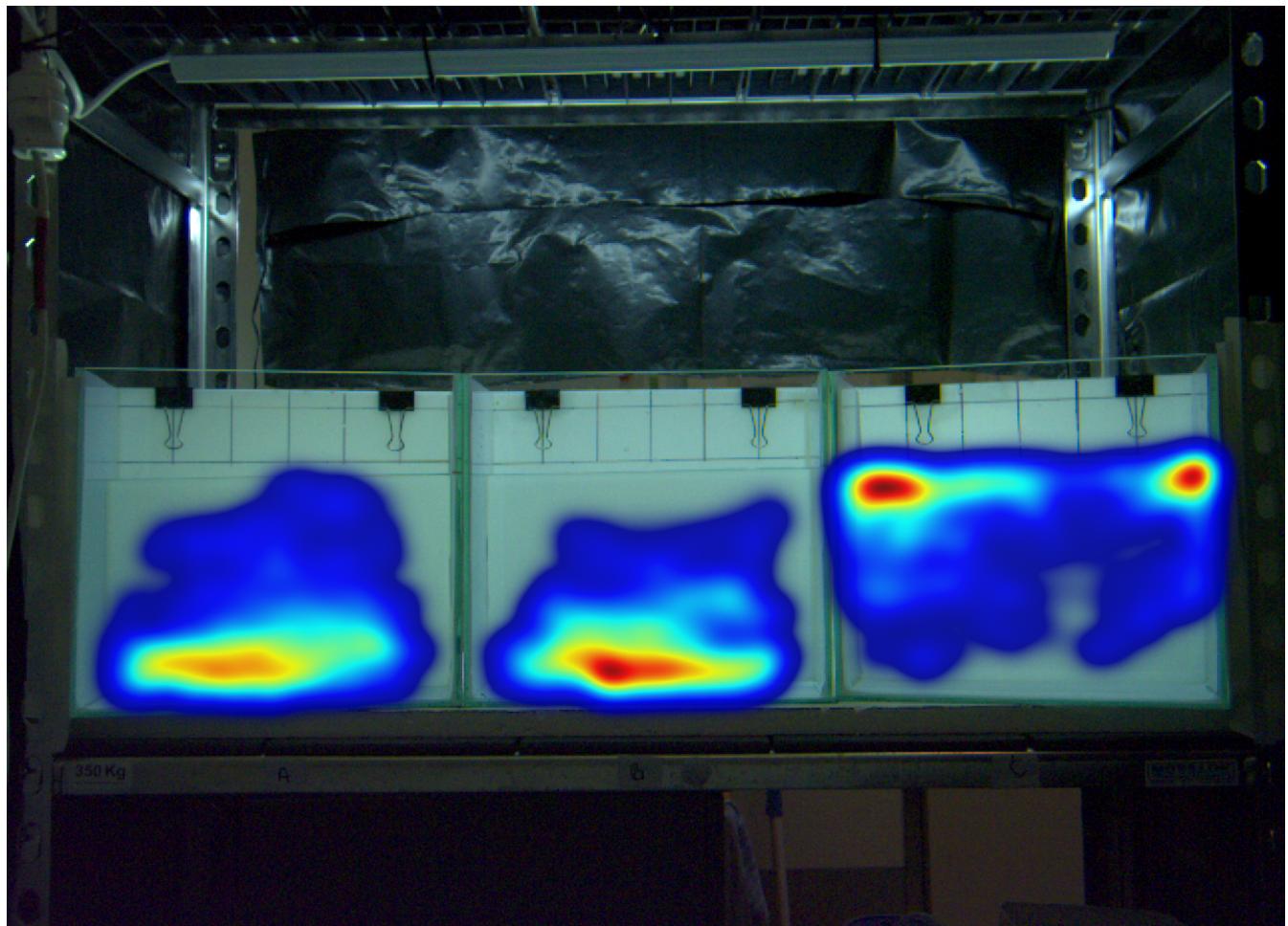


Figure 3.3.1: Range, median, and upper and lower quartiles of anxiety levels before treatment, measured as the percent of a 10-minute trial spent in the bottom portion of the tank (anxious n = 62, non-anxious n = 27).



*Figure 3.3.2:* Heatmap showing how three fish used the space in their tanks during phenotyping. The first two fish were anxious, and it is clear that they spent a lot of time in the bottom part of the tank. In comparison, the fish in the right-most tank was non-anxious; he spent less time in the bottom and more time swimming in the rest of the tank, especially toward the top corners.

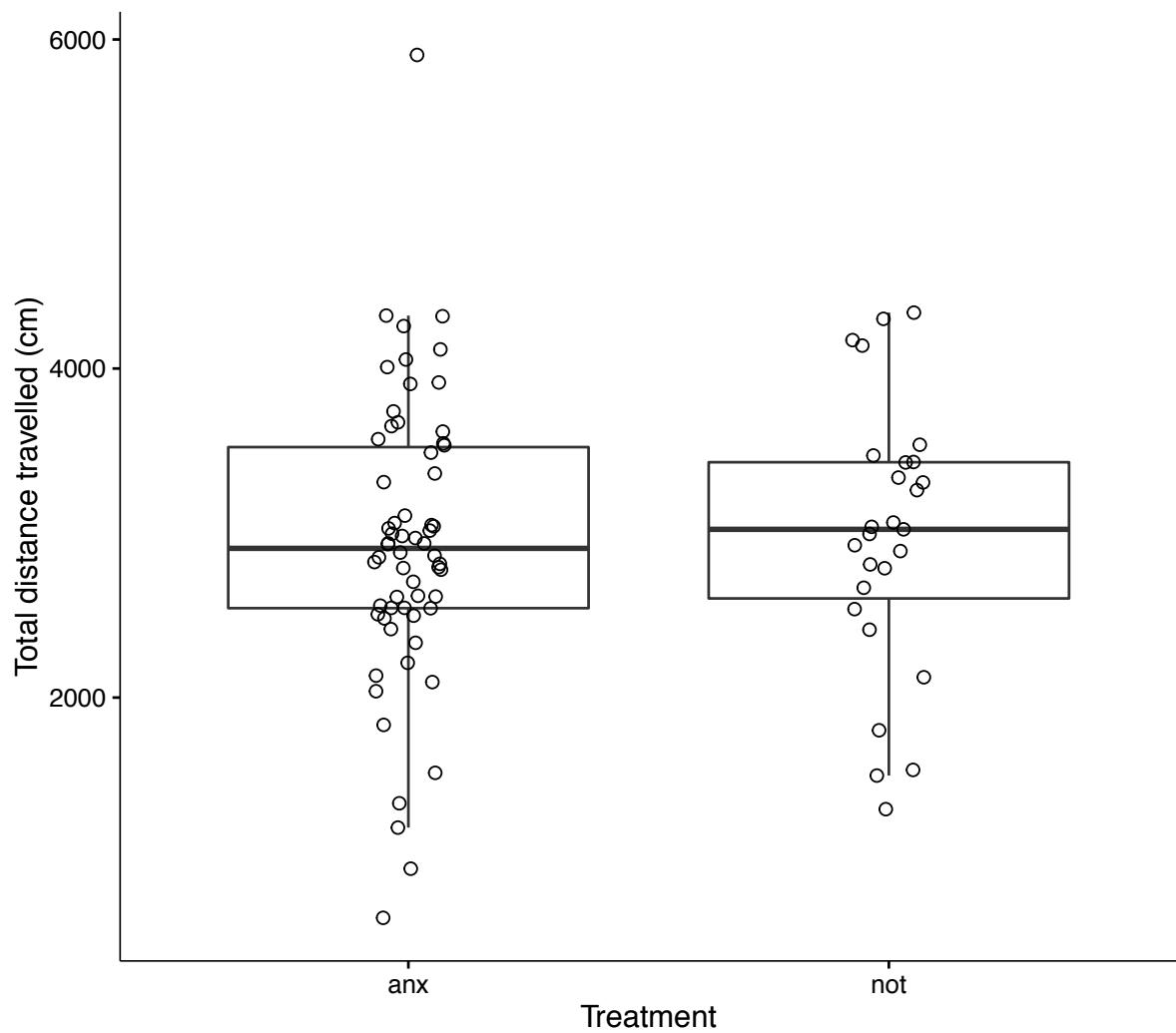


Figure 3.3.3: Range, median, and upper and lower quartiles of distance travelled by anxious ( $n = 62$ ) and non-anxious ( $n = 27$ ) fish during initial phenotyping before treatment.

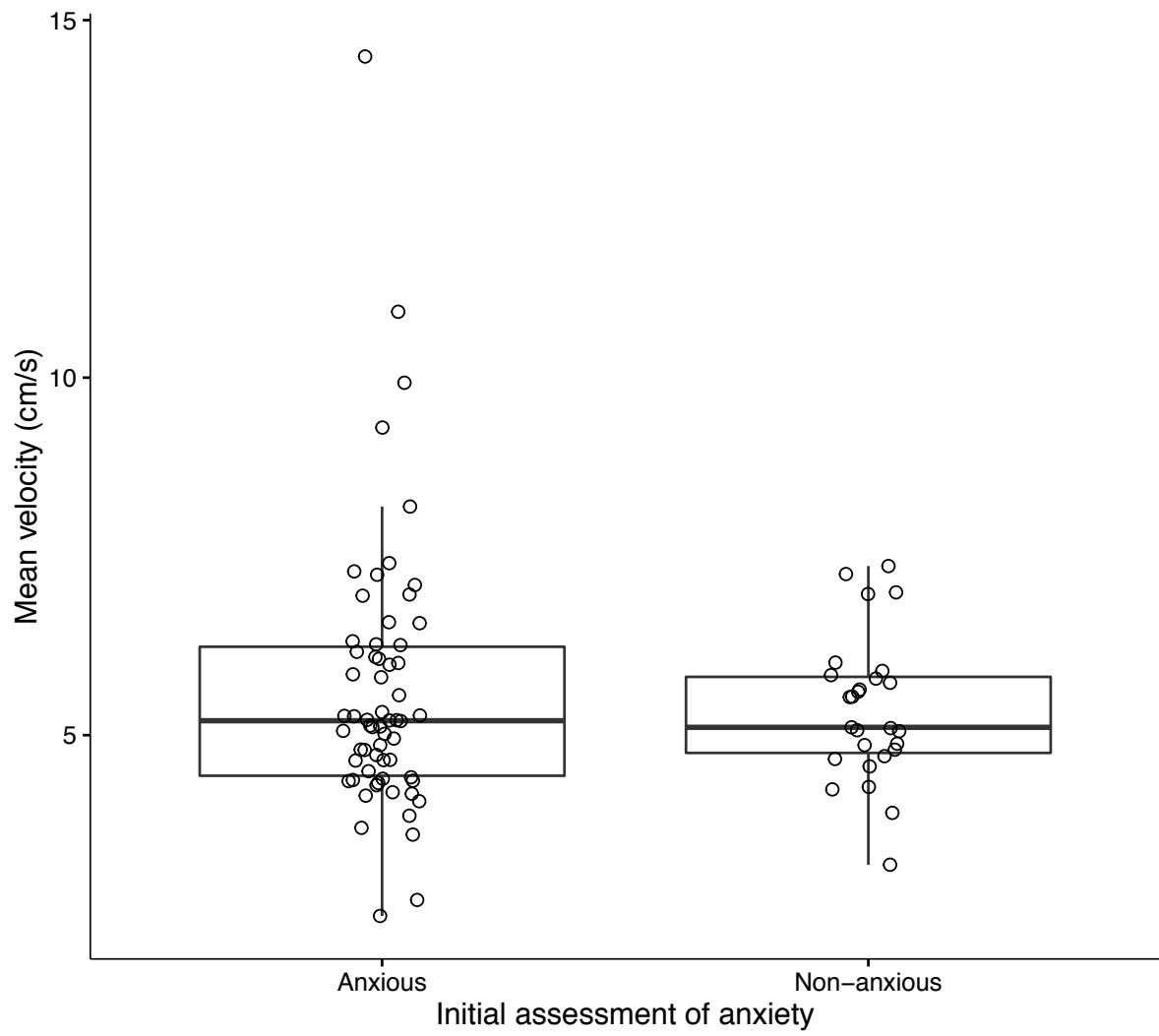
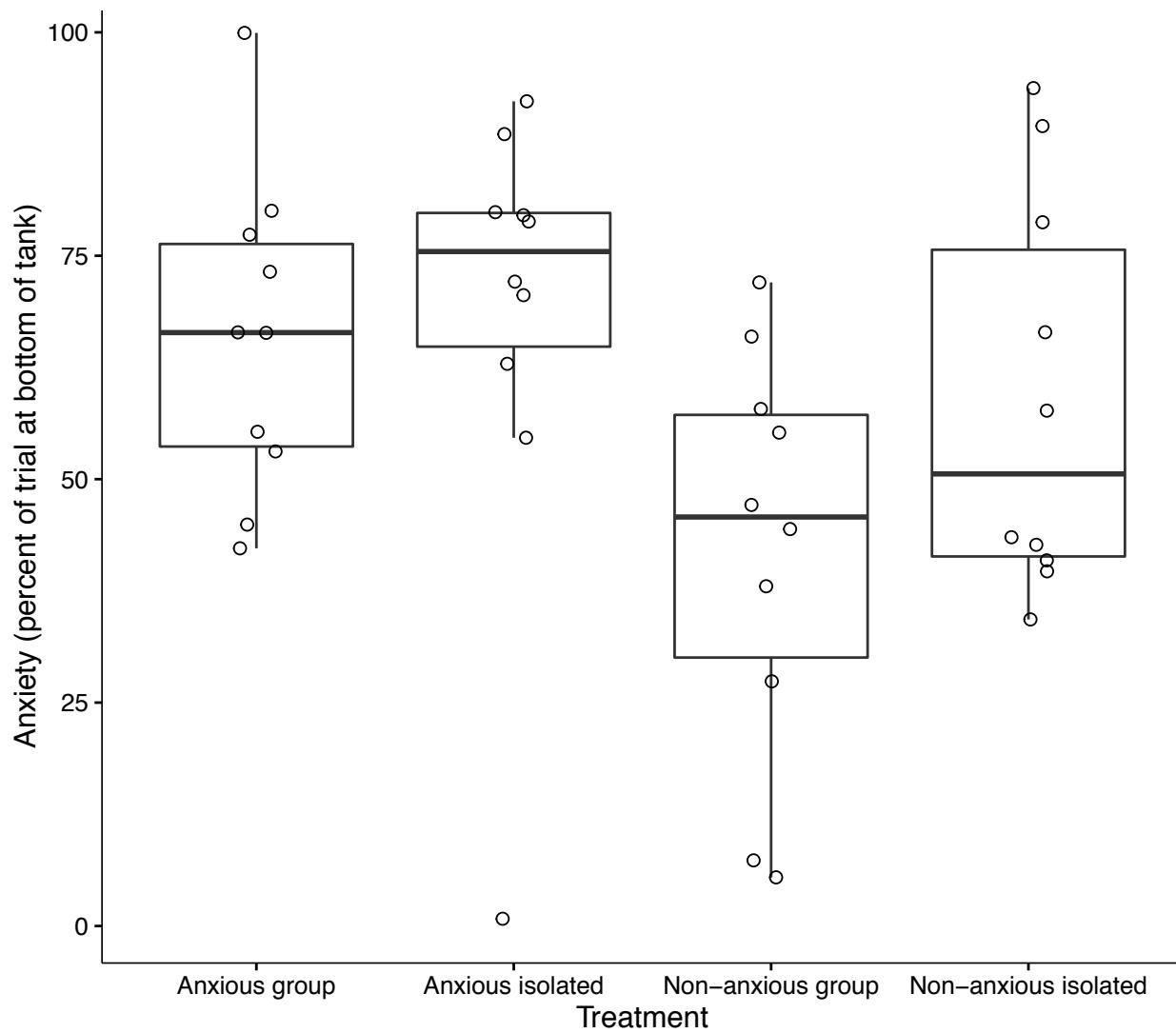


Figure 3.3.4: Range, median, and upper and lower quartiles of velocity in cm/s during initial phenotyping (anxious n = 62, non-anxious n = 27).

### ***3.3.1 Anxiety levels post-treatment***

While there was wide variation in some treatment groups (Fig. 3.3.5), after a 21-day exposure to group-living or social isolation, non-anxious fish remained less anxious than the fish that were phenotyped as anxious before the treatment ( $t = -2.37$ ,  $p = 0.023$ ; Fig. 3.3.1 c.f. Fig. 3.3.5). However, whether a fish was isolated or housed in a group had no significant effect on anxiety ( $t = 1.03$ ,  $p = 0.31$ ; Fig. 3.3.5), and there was no interaction between initial anxiety level and treatment ( $t = 14.5$ ,  $p = 0.32$ ). That said, some patterns are evident; for example, anxiety levels tended to change after the three-week period for both group-housed and isolated fish. The initially anxious fish became less anxious, but remained in the anxious spectrum, while the non-anxious fish became more anxious; in particular, the isolated non-anxious fish crossed into the anxious side of the spectrum. The Cohen's d effect sizes were all large and significant and mirrored the patterns observable in the data; the anxious group-housed and isolated fish both showed large, negative differences of -1.62 (95% CI = -2.34, -0.90) and -1.22 (95% CI = -1.92, -0.52) respectively, and, for the non-anxious group-housed fish, the magnitude of difference was similar but positive (1.09; 95% CI = 0.32, 1.86). In the non-anxious isolated fish, however, the magnitude of difference between the initial anxiety levels and post-treatment anxiety levels (2.22; 95% CI = 1.33, 3.10) was almost twice as high as for the other three groups.

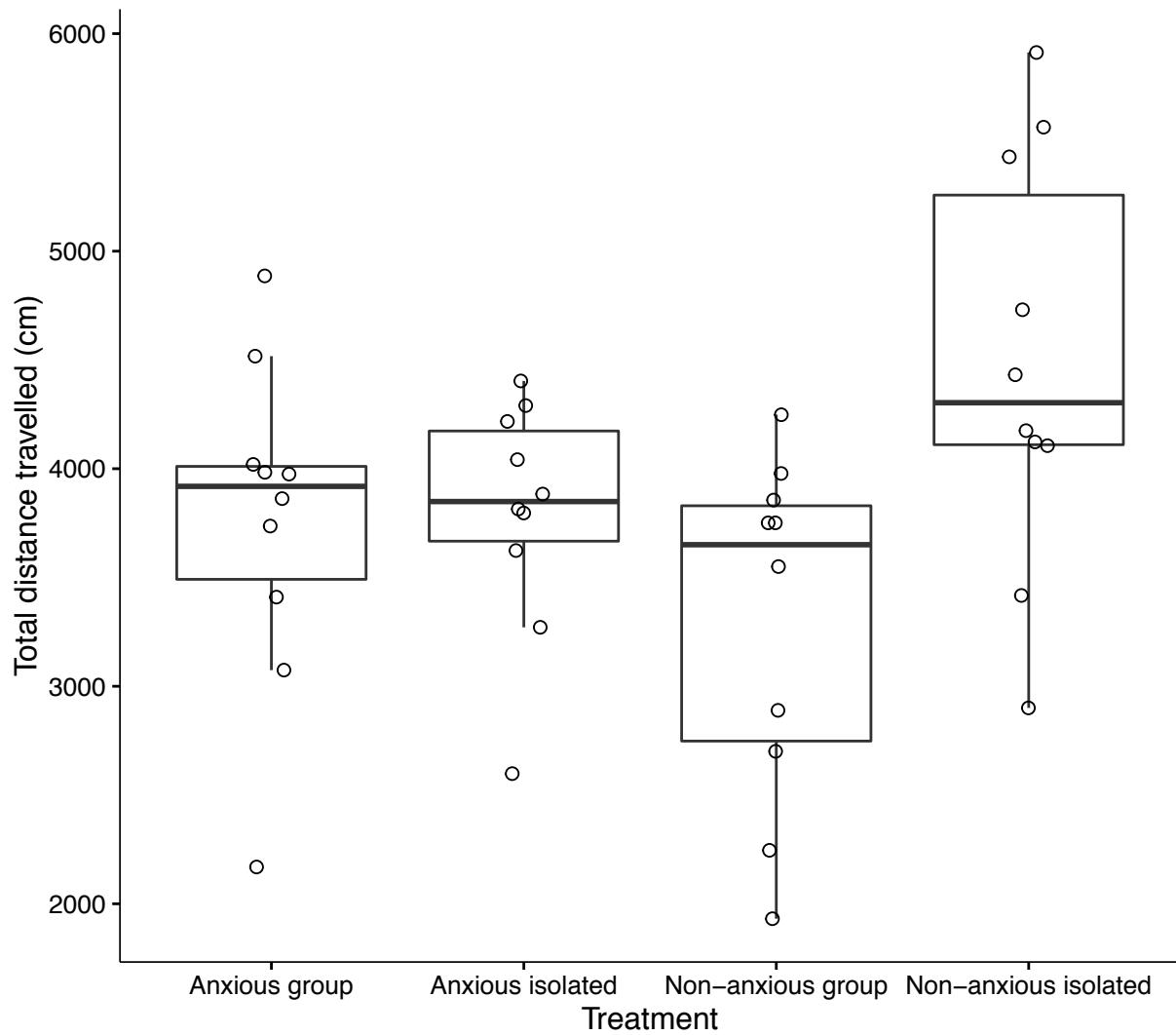


*Figure 3.3.5:* The range, median, and upper and lower quartiles of anxiety levels (measured as percent of time spent in the bottom of a tank) shown by initially anxious and non-anxious fish after group-housing or isolation treatments ( $n= 10$  in each group).

### ***3.3.2 Distance and velocity post-treatment***

Neither treatment ( $t = 0.09$ ,  $p = 0.93$ ) nor initial anxiety ( $t = -1.37$ ,  $p = 0.18$ ) were significantly associated with the distance travelled during phenotyping, but the interaction between treatment and initial anxiety was significant ( $t = 2.37$ ,  $p = 0.023$ ), indicating that the effects of social isolation on distance travelled were different for the initially anxious and non-anxious fish. The effect sizes were all significant and high when the initial distances (Fig. 3.3.3) and post-treatment distances (Fig. 3.3.6) were compared. The anxious group-housed and anxious isolated treatment groups showed effect sizes of 1.02 (95% CI = 0.336, 1.71) and 1.09 (95% CI = 0.401, 1.78) respectively. The non-anxious group-housed fish showed a similar difference after treatment (1.18; 95% CI = 0.405, 1.95), while the non-anxious isolated fish again showed a higher effect size of treatment, with 1.68 (95% CI = 0.863, 2.51).

Similarly, neither treatment (GLM  $t = -1.4$ ,  $p = 0.17$ ) nor initial anxiety ( $t = 0.08$ ,  $p = 0.94$ ) were significantly associated with the velocity, but there was a significant interaction between treatment and initial anxiety (GLM  $t = 2.38$ ,  $p = 0.023$ ). The effect sizes for the changes in velocity from the initial phenotyping (Fig. 3.3.4) to the post-treatment phenotyping (Fig. 3.3.7) show that the only group whose mean velocity changed significantly was the non-anxious isolated group, which was (on average) faster after treatment; the effect size for this group was 1.8 (95% CI = 0.97, 2.64), which is both large and significant. Both of the anxious treatments and the non-anxious isolated treatment had fairly low, non-significant effect sizes for the differences in velocity (0.37 (95% CI = -0.30, 1.0), 0.40 (95% CI = -0.28, 1.1), and 0.13 (95% CI = -0.60, 0.86) respectively).



*Figure 3.3.6:* The range, median, and upper and lower quartiles of total distance travelled in cm during a trial by initially anxious and non-anxious group-housed and isolated fish. n = 10 for each treatment group.

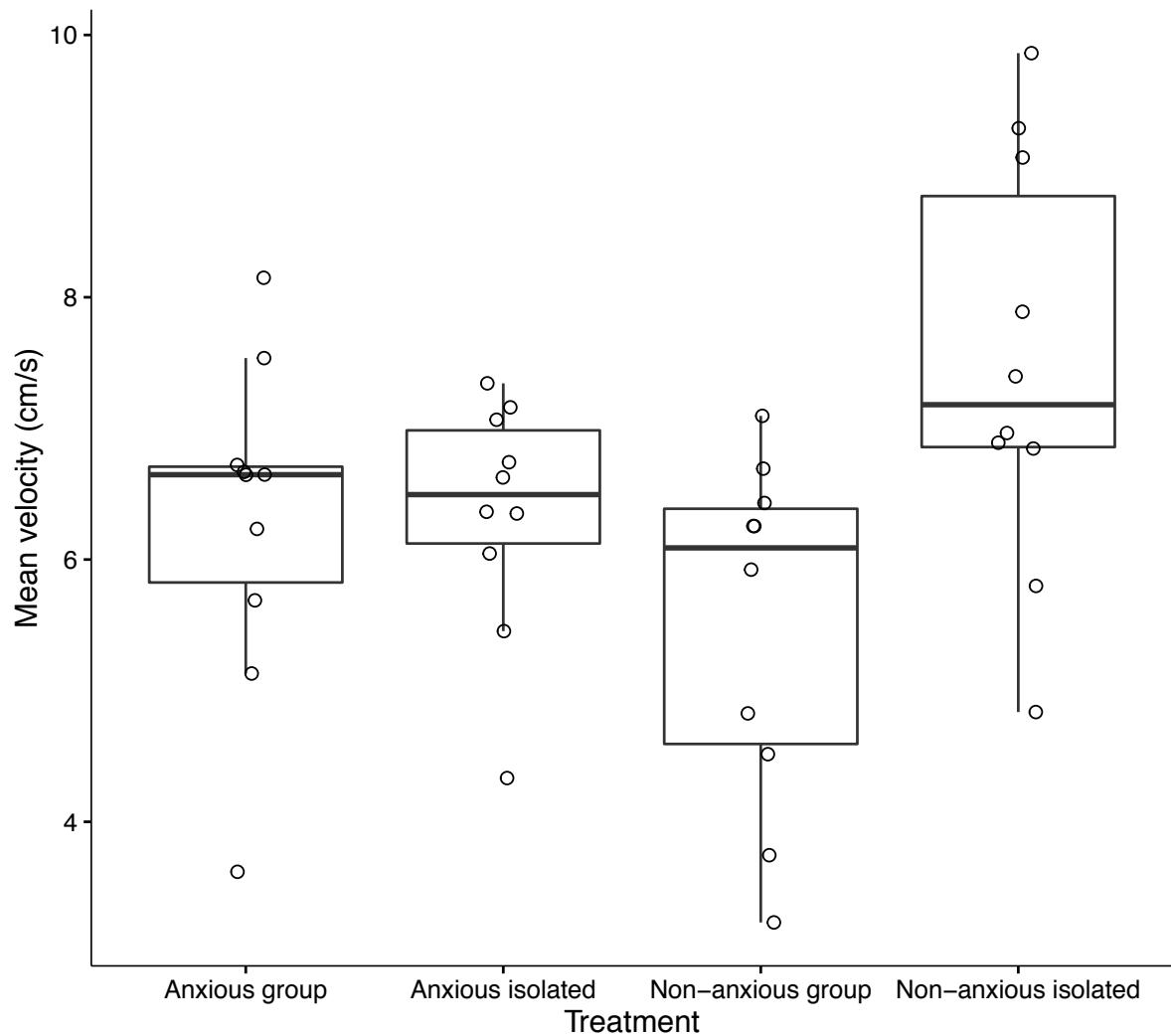


Figure 3.3.7: Range, median, and upper and lower quartiles of mean velocity in cm/s for each treatment group ( $n=10$  per group) after the treatment period.

## **3.4 Discussion**

The main aim of this study was to test whether initially anxious and non-anxious fish show changes in anxiety levels after a period of social isolation. Both isolated and group-housed anxious fish became less anxious on average after the test period, while both of the non-anxious test groups became more anxious. The largest change in anxiety levels, however, was seen in the non-anxious isolated fish; their mean change in anxiety was roughly double that of the other test groups. The non-anxious isolated treatment group also showed the greatest change in distance travelled and mean velocity of all the other treatment groups. This indicates that social isolation influenced the anxiety levels and other activity-related behaviours of the non-anxious fish more than the anxious fish.

### ***3.4.1 Anxiety***

The social isolation experiment demonstrates that whether the fish were in a group or isolated for the three weeks did not affect their anxiety levels, but whether the fish were anxious or non-anxious before the experimental period predicted anxiety. Despite increases in anxiety in the non-anxious fish and decreases in the anxious fish, the initially non-anxious fish remained less anxious on average after the treatment period than the initially anxious fish. However, because the fish were not individually tagged, I could not run a paired analysis to see how much each individual fish changed its behaviour. The model therefore did not include the before values, using only whether a fish was previously phenotyped as anxious or non-anxious. Considering the effect sizes, however (all of which showed large and significant standardised differences between the mean before anxiety values and post-treatment anxiety values), it appears that behaviour did change after the treatment period. Comparing the before and after mean anxiety levels demonstrates large and significant effects, with group and isolated anxious fish experiencing a decrease in anxiety of 18.3% and 16.2% respectively. By

contrast, the non-anxious group fish anxiety increased by 16.1%, and the non-anxious isolated fish anxiety increased by 32.7%. Thus, while the anxious fish and the non-anxious group-housed fish all remained on the side of the anxiety spectrum on which they started, the non-anxious isolated fish were the only treatment group to move into the other half of the spectrum, becoming significantly more anxious. In a previous study on social isolation of zebrafish, three weeks of isolation led to an overall increase in anxious behaviour, measured as the number of transitions between the upper and lower portions of the tank, but not in the time spent in the bottom (Collymore et al. 2015). However, Collymore et al. (2015) divided the tank in half to get the upper and lower portions, which may have affected the way they measured anxiety; we know from other studies that the amount of time spent at the bottom of the tank does increase in response to anxiogenics, and it decreases with anxiolytics (Levin et al. 2007; Bencan et al. 2009; Egan et al. 2009). The specifics of the effects of social isolation on anxiety levels are somewhat unclear; however, Parker et al. (2012) found that isolated fish were less anxious and had lower cortisol levels, perhaps because of a lack of feedback or conformity effect from conspecifics. It may be that anxious fish are better able to cope with social isolation. Regardless, it seems likely that there was a real effect of isolation on the behaviour of previously non-anxious fish, but the power of my study was perhaps not high enough to detect this effect because of the small sample size.

Prior to the experiment, the groups defined as anxious and non-anxious clearly showed different mean levels of anxiety (Fig. 3.3.1). The scale which I devised to estimate zebrafish anxiety using the time in the bottom of the tank was bottom-heavy, but it is clear from the ratios of anxious to non-anxious categorisations in the initial phenotyping (62 anxious to 27 non-anxious) that this was necessary because zebrafish are naturally anxious. I wanted to account for that and identify fish that were non-anxious relative to other zebrafish. The mean

proportion of time spent in the bottom across all of the zebrafish I initially phenotyped was 61.3%, which would count as somewhat anxious on my scale. This is very similar to the zebrafish from the repeatability experiment (Chapter 2), whose mean was 64.3%, including all test points from the initial test to the week 27 repeat test. Thus, the fish from the present experiment had similar anxiety levels to those from the repeatability study, and so the scale I devised is likely to have been appropriate for this cohort of fish.

The interaction of treatment and initial anxiety was significant for both distance travelled and mean velocity; the isolated non-anxious fish moved greater cumulative distances and at a higher speed than the other treatment groups. The effect sizes for distance tell a similar story to the effect sizes for anxiety; all of the treatment groups had significant, fairly high effect sizes, but the effect size of non-anxious isolated fish was larger than the others. For velocity, however, the only statistically significant effect size was for the non-anxious isolated fish; this indicates that this treatment group was the only one to show a real effect of the three week treatment period on mean velocity. In rats, living in isolation has been linked to higher activity levels (Cheeta et al. 2001); my findings support there being a similar effect in previously non-anxious zebrafish. Activity levels may be linked to anxious behaviour; zebrafish often exhibit fast, erratic swimming behaviour when alarmed (Parra et al. 2009; Kalueff et al. 2013). Cocaine withdrawal symptoms in zebrafish are similar to those in other vertebrates, and include increased anxiety and hyperactivity (López-Patiño et al. 2008). The treatment group which showed the greatest increase in anxiety (the non-anxious isolated fish) also showed the greatest increase in mean velocity, which further supports the idea that anxiety and activity in zebrafish may be linked.

### ***3.4.2 Factors that can alter behaviour***

There are a multitude of factors which could have contributed to the unexpected changes in behaviour by the anxious fish (that is, both isolated and group-housed fish becoming less anxious). It must be noted that the fish were initially phenotyped during their first experience in the test tanks. The percent of time spent in the bottom of the tank during the first ten minutes was found to be repeatable overall in Chapter 2 of this thesis ( $R = 0.353$ ; 95% CI = 0.214, 0.517), but it was not repeatable between the initial test and the one-week ( $R = 0.107$ ; 95% CI = -0.187, 0.385) or five-week ( $R = 0.198$ ; 95% CI = -0.096, 0.461) repeats. In the discussion of that experiment, I suggested that this may have been because of a need for the fish to have an initial experience in the test tanks before displaying their natural behaviour. Accordingly, the percent of time spent in the bottom of the tank was repeatable between the one-week and five-week repeats ( $R = 0.442$ ; 95% CI = 0.176, 0.649). Anxiety generally appeared to decrease over time, but the difference between week 1 and week 5 was small (mean 12.7% higher in the initial trial than week 5, but only 1.6% higher in week 1 than week 5). Because an initial pre-phenotyping exposure was not done in this study, some of the fish may have been incorrectly categorised as anxious or non-anxious at the start of the experiment, and this might thus contribute to some of the unexpected changes in anxiety levels in the group-housed fish, and especially in both of the anxious treatment groups, where anxiety was lower on average after the test period. Additionally, the initial phenotyping was the first time the fish had been handled after being transported to the Zoology department from the Otago Zebrafish Facility, and so this may have further affected their behaviour. However, the differences between initial and post-treatment anxiety in this experiment were larger than in the repeatability experiment, ranging from 16.1% higher for the non-anxious group-housed fish to 32.7% higher for the non-anxious isolated fish. Hence, the behavioural

changes were larger after a change in context than they were over time, and so the treatment appears to have had an effect on anxiety levels.

An issue that was noted with the group-housed fish was that, in groups of five, the fish appeared to behave much more aggressively than usual. Levels of aggression were not measured, but it was noted during daily feedings that the fish appeared to be chasing each other more than is normally observed in a more densely-stocked tank. A study that investigated stocking density found that zebrafish prefer to be stocked at about ten fish per two litres of water, with no evidence of aggressive behaviour at this density (Pavlidis et al. 2013). It also demonstrated that body cortisol levels are higher in zebrafish kept in small groups because of the formation of dominance hierarchies; in one litre tanks in dyads, dominant males can spend 21-61% of the time chasing the subordinate males, while, in groups of five, the dominant fish may spend 24-41% of the time chasing. Furthermore, the dominant individual in a dyad may bite the subordinate, and maintain exclusive access to the upper portions of the tank (Theodoridi et al. 2017). The test fish in the present study were stocked in groups of five in 3.25 litres, which is less than half the recommended stocking density; thus, this may have contributed to why the non-anxious fish kept in groups experienced an increase in anxiety during the 21-day exposure.

Dominance hierarchies are extremely common both in natural environments and in the laboratory; they often involve one or more individuals behaving aggressively toward others to establish and maintain their position of dominance (Drews 1993; Sloman and Armstrong 2002). Social dominance has been observed in both male and female zebrafish. Larger individuals are often the dominant ones, with generally better reproductive outcomes for the dominant fish, especially in males (Paull et al. 2010). The zebrafish in the group treatments

for the current study were kept in mixed-sex groups, and some of the tagged individuals added to make up groups were smaller than others; the high levels of aggression could have been related to competition for mates and differences in body size, as well as the small group sizes. A study on male Australian field crickets (*Teleogryllus oceanicus*) found that changes in dominance status (for example, a previously subordinate individual becoming more dominant) can change the expression of personality traits including boldness, activity, and exploration (Rudin et al. 2016). If this also happens in zebrafish, the levels of aggression seen during the three-week treatment periods may have been due to the establishment of dominance hierarchies, which may have changed the behaviours of the zebrafish when they were tested after the experiment. In the cricket study, subordinates that became dominant became bolder and more active, while dominants that became subordinate became less bold and less active (Rudin et al. 2016). Aspects of behaviour which change according to dominance status may, however, depend on the presence of the dominant or subordinate counterpart, rather than reflecting a permanent change (Blanchard et al. 2001).

Any change in dominance status has been shown to reduce the repeatability of activity-related behaviour, but not of boldness in crickets (Rudin et al. 2016). In my experiment, the variance in anxiety after the experimental period (Fig. 3.3.3) was higher than in the initial phenotyping (Fig. 3.3.1), with a few individuals whose anxiety levels were notably different from the group mean post-treatment. In the isolated anxious fish, all values were between 60 and 90%, except for one individual which spent just 0.8% of its time in the bottom. The group non-anxious fish were mostly in the 30 to 60% range, but two individuals spent only 5 and 7% of their time at the bottom. In the isolated non-anxious group, there were no values below 30%. The small sample size of my study, with only ten individuals in each treatment group, unfortunately means that these extreme values could have had a fairly large effect on

the statistical comparisons. Paired before and after data, where each individual's repeatability in anxious behaviour could be calculated, would have made more detailed analysis possible. The effect size values, however, take into account the different standard deviations and sample sizes between the initial tests and the after tests, and so they are a good indicator that the patterns observable in the data are real.

There are a number of other factors which would affect the behaviour of zebrafish in tests such as this even if there were a large sample size and paired before-and-after data. A non-novel tank reduces the tank diving response (Bencan et al. 2009) and several re-exposures to a novel tank over the course of seven days result in less erratic movement and freezing, and more exploration of the tank, particularly after the first few exposures (Wong et al. 2010). The zebrafish in my study were exposed to the test tanks only twice, and the post-treatment phenotyping was done between four and eleven weeks after the initial phenotyping, so habituation is unlikely to have been an issue. I did note, however, that most of the measures associated with activity (as well as anxiety) in the repeatability experiment (Chapter 2) were more repeatable after a second experience in the novel arena than after just one experience. Nevertheless, the repeatability experiment did show that the percent of time at the bottom during the novel arena test was repeatable overall, which would not be the case if the initial test had been completely different.

In the future, it would be interesting to carry out a similar experiment to the present study, but return the zebrafish to groups afterwards. The fish could then be phenotyped again after a further three weeks of group living, to determine whether their behaviour would return to the baseline means, or whether the changes observed were longer-lasting. Unfortunately, limitations of space and time meant that I was unable to attempt this extension of my social

isolation study. Another possibility for further investigation is that the social isolation treatment could be performed at different life stages; this relates to the “critical period” life course model (Nicolau et al. 2007), in which previous research that suggests stress has the largest effect on behaviour when it happens at a young age (Caldji et al. 2000; Teicher et al. 2006; Steenbergen et al. 2011). It could be thus determined whether a similar pattern of decreasing effects of social isolation stress with age is seen with social isolation in zebrafish. Zebrafish learn their preferred associations from early-life exposure to conspecifics (Spence et al. 2008), and so we might expect that isolation as fry would have a larger effect on behaviour than isolation as adults.

Different strains of zebrafish show different anxiety responses in behavioural tests (Lima et al. 2016); some strains have intrinsically higher levels of anxiety than others, but all the strains tested in one study had similar activity levels (Egan et al. 2009). The strain used in the current experiment was a wild-type AB, which Egan et al. (2009) identified as a relatively low-anxiety strain. In both the repeatability and the social isolation experiments in this thesis, the average proportion of time spent in the bottom was around 60%, while Levin et al. (2007) stated that zebrafish generally spend 55% of the first 5 minutes at the bottom of a novel tank. However, these two measures are similar when potential measurement error is considered. It was not stated which strain of zebrafish they used. In my own experience, there can be within-strain differences in behaviour. In several preliminary trials, I found that conspecific alarm cue would consistently elicit the expected anxious response from some AB fish, but only occasional responses from other AB fish, the difference being that some had been brought into the Otago Zebrafish Facility for breeding several years (and many generations) before the others. The applicability of specific responses rather than general trends to other strains or populations may be limited; for example, populations of a tropical poeciliid fish

(*Brachyraphis episopi*) from areas of high predation pressure are bolder than fish of the same species from areas of lower predation (Brown et al. 2005). Thus, while the stress responses of zebrafish are similar enough to other vertebrates that their physiological and behavioural responses to challenges can be broadly applied, even to humans and other mammals (Steenbergen et al. 2011), care must be taken when applying specific findings from one study to another.

Although there are many different animals which show some degree of sociality, there have been comparatively few studies done on the effects of social isolation in systems other than commonly-used model organisms like rodents and zebrafish. Studies with rhesus monkeys (*Macaca mulatta*) have made important contributions to social isolation research; monkeys raised in total social isolation perform repetitive movements, hostility toward others and self, and an inability to form social bonds when allowed to interact with conspecifics (Harlow et al. 1965). Dairy cows (*Bos taurus*) exhibit stereotypical anxious behaviours as well as elevated saliva cortisol levels when separated from conspecifics for a period of just 20 minutes (Müller and Schrader 2005). Asian elephants (*Elephas maximus*) also exhibit stereotypic behaviours when kept isolated in captivity (Vanitha et al. 2011). Socially monogamous prairie voles (*Microtus ochrogaster*) display depression-like behavioural and neuroendocrine symptoms when social isolation is combined with stressors like resident-intruder tests (Grippo et al. 2007), and rats reared in isolation are more aggressive toward conspecifics than rats reared socially (Wongwitdecha and Marsden 1996). However, most examples in the literature fit into the broad categories of primate and rodent. More research must be carried out on the effects of social isolation on social non-model organisms in order to identify whether the patterns which have thus far been observed in model and domestic species are more widely applicable across the animal kingdom.

There is evidence that social isolation can also change the way in which anxiogenics and anxiolytics work. The anxiolytic drug diazepam decreases active interactions in isolated rats but not group-reared rats (Wongwitdecha and Marsden 1996). A study of the effects of nicotine on social interaction found that anxiolytic effects were seen at a wider range of doses in socially isolated rats than in group-housed rats in a social interaction test, and not at all in an elevated plus maze test (Cheeta et al. 2001). It may be important for human health that further study be done on the interaction between social isolation and various drugs.

Accordingly, alcohol is a drug commonly studied in relation to anxiety. There are also documented anxiolytic effects of alcohol on humans. Moderate alcohol consumption lowers reported anxiety levels as well as the rate of panic attacks in response to a panic challenge in people with panic disorder, while higher alcohol intake returns anxiety levels to baseline (Williams 1966; Kushner et al. 1996). Alcohol activates reward circuits in the brain while reducing fearful responses to visual stimuli (Gilman et al. 2008). Many factors contribute to habitual alcohol abuse, but a major one of these is thought to be perceived social isolation (Åkerlind and Hörnquist 1992). This may be partially due to the different effects of alcohol on socially isolated individuals. For example, ethanol has shown anxiolytic effects during a novel tank diving test on individually housed zebrafish, but not on group-housed ones (Parker et al. 2012). It has also been shown in monkeys that early-life experiences such as social isolation, which predispose to fearful behaviour, contribute to greater voluntary consumption of alcohol (Higley et al. 1991). Similarly, socially isolated rats show more anxious behaviour and higher cortisol levels, as well as a preference for ethanol over water. These associations were not seen in group-housed rats exposed to the same tests (Butler et al. 2013). Thus, it may be that, along with other negative health effects (see Cole et al. 2007; Hawley and Cacioppo 2010; Luo et al. 2012), greater susceptibility to alcohol abuse may also contribute to the adverse health outcomes of social isolation. The wide range of effects of social

isolation on humans and on other animals demonstrates why it is important that this phenomenon be studied.

### ***3.4.3 Conclusions***

This study aimed to investigate whether the effects of social isolation differed between individuals previously identified as anxious or non-anxious, and whether a period of social isolation was indeed enough of a stressor to disrupt a repeatable behaviour. I predicted that the non-anxious isolated fish would become more anxious, and that there would be little change among the other test groups. Overall, the findings of my study support the idea that separation from conspecifics can change behaviour, and that prior anxiety can alter the behavioural effects of social isolation in zebrafish. While few statistically significant differences were found, the high effect sizes of the differences between initial anxiety levels and the post-treatment values in this study imply biological effects of social isolation, despite low statistical power. The lower post-treatment levels of anxious behaviour in the initially anxious fish may have been because of a lack of experience in the test tanks, or just from variable results and low sample sizes (although, because of the high effect sizes, this seems unlikely). The previously non-anxious isolated fish experienced increased anxiety and activity-related behaviour, matching my initial predictions. This appears to be a real effect of social isolation, especially considering the significant change in anxiety over the other three treatment groups. The changes in distance travelled and mean velocity during the trials also imply an effect on activity.

# **Chapter Four**

## **General Discussion**

The main aim of this thesis was to examine the consistency of behaviours over time and across contexts. I did this in two parts: a repeatability experiment and a social isolation experiment. The aims of the repeatability experiment (Chapter 2) were to test whether a suite of personality-related behaviours were repeatable in individuals over a 6-month period, as well as to identify any sex differences in either repeatability or the behaviours themselves.

The final aim of this experiment was to test for associations among the behaviours studied, the existence of which would suggest the presence of behavioural syndromes. In the social isolation experiment (Chapter 3), I aimed to examine whether a period of social isolation was sufficiently challenging to disrupt the consistency of anxious behaviour in individuals, and also to determine whether activity levels were linked to anxiety.

For the experiment in Chapter 1, I predicted that behaviours would be repeatable overall. I also predicted that males would have more repeatable behaviour, and that there would be evidence of a behavioural syndrome. Conversely, for the experiment in Chapter 2, I predicted that a period of social isolation would be enough of a challenge to alter behavioural

expression of anxiety, and disrupt the consistency of this behaviour. I also predicted that activity levels would change proportionately with anxiety.

I found that activity, exploration, and anxiety were repeatable overall, as was sociability at the tank level. Boldness was also repeatable, but this was only just significant. I observed sex differences in distance travelled (activity) during the novel arena assay, and in novel object approach (boldness) during the novel object assay, as well as in the mean distance to the nearest neighbour during the sociability test. There was evidence of an activity-exploration-boldness behavioural syndrome but, because the activity and the exploration measures were taken from the same footage, the association between these two traits is non-independent. In the social isolation experiment, I found that, while all four treatment groups exhibited changes in anxiety levels, it was indeed the initially non-anxious isolated fish which experienced the greatest mean increase in anxiety. Both the group-housed and the isolated anxious fish became (on average) somewhat less anxious, and the non-anxious group-housed fish became somewhat more anxious.

## 4.1 Repeatability over time

Overall, my hypothesis that personality-related behaviours would be repeatable in a cohort of zebrafish over 6 months is supported by my findings. This also supports the aspect of the common definition of animal personality which states that behaviours are consistent over time (Dall et al. 2004; Sih et al. 2004a; Réale et al. 2007). The findings from this study are important because they demonstrate that these behaviours are repeatable over a longer period of time than is generally tested; in Table 1.1, 12 of 17 studies used test intervals ranging from within 24 hours to two weeks, and the largest test interval was three months. The longest repeatability interval of which I am aware is that of David et al. (2012), who gave

repeatability estimates for birds over a 7-month interval, but this is highly unusual within the literature. A potentially important finding from my repeatability study which I have not come across in previous studies was that the behaviour of the zebrafish was more repeatable from the one-week trial than from the initial trial. This was unexpected, because shorter intervals between tests usually produce higher estimates of repeatability (Bell et al. 2009; David et al. 2012). A study on the consistency of activity with zebrafish tested daily over 7 days did, however, find that activity levels were highest on the first day of testing, but the researchers attributed this to habituation because of the large number of tests conducted across short intervals (Tran and Gerlai 2013). It is unlikely that there was much effect of habituation on the fish in my repeatability study though, because the test intervals were wide, except between the start and the week 1 test. It may have been more that the fish were too anxious to express their natural behaviour in the initial test; anxiety levels measured as the percent of time in the bottom of the tank were higher in the initial test than the one week test. The initial test was the first time the fish had encountered the test tanks, and also the first time they had been handled since being transferred to the Zoology Department from the Otago Zebrafish Facility. The fish may thus have been more stressed during this test than in subsequent tests.

Most of the repeatability estimates from this experiment are in line with what other researchers have found using similar tests. In a sample of the existing literature on repeatability of personality-related behaviours (Table 1.1), the range of repeatability values was from -0.03 to 0.68; however, the high value is from a study with a sample size of just eight individuals (Carlson and Langkilde 2013). Excluding this, the range in the Table is from -0.03 to 0.59. All of my repeatability estimates lie within this range, but it should be noted that most repeatability values in the Table of 0.2 or below were not significantly repeatable. The two boldness measures in my study had repeatability estimates of ~0.18, but

they were both significant (Fig. 2.3.5), and so it again appears that some of the previous studies suffer for their small sample sizes. Comparing the literature surveyed in my table, for which the combined mean repeatability estimate is 0.37, with the meta-analysis by Bell et al. (2009), which produced an identical mean estimate of 0.37, it appears that the repeatability estimates I produced for activity (0.49 and 0.26), anxiety (0.35) and exploration (0.35) are congruent with the existing literature.

## 4.2 Consistency across contexts

The findings from the social isolation study mostly supported my hypothesis that social isolation would disrupt the consistency of a repeatable behaviour, in that the non-anxious isolated fish showed the greatest change in anxious behaviour, by becoming much more anxious after treatment. The initially anxious fish, however, from both isolated and group-housed treatments, showed unexpected responses by becoming less anxious. The issue with higher repeatability from the week 1 repeat than from the initial week may have contributed to these findings. Additionally, it was also this cohort's first time being handled since transfer from the Otago Zebrafish Facility. Thus, some of the fish may have initially been phenotyped incorrectly. The non-anxious isolated fish showed the greatest increases not only in anxiety but also in activity, which supports the known link between anxious and hyperactive behaviours in zebrafish (López-Patiño et al. 2008; Egan et al. 2009; Gerlai 2013).

Isolated zebrafish are more stressed and have higher variability in behaviour than groups of fish (Pagnussat et al. 2013). Visual contact with conspecifics triggers reward pathways in zebrafish, and has been used as a reward in associative learning (Al-Imari and Gerlai 2008). While increased levels of anxiety after social isolation for three weeks have been demonstrated (Collymore et al. 2015), a study found that anxious behaviour decreased when

fish were kept isolated from conspecifics for 90 days (Shams et al. 2015). These two studies kept the fish isolated for different lengths of time (the Shams et al. study for more than four times the length of the Collymore et al study), which could have contributed to the differences in findings. However, reduction of the anxious response after social isolation was seen by Parker et al. (2012) after just two weeks. Similarly, Giacomini et al. (2015) isolated zebrafish for 15 and 30 days and found that the cortisol response did not differ between the 15 day and the 30 day treatments, but (on average) the isolated fish had a higher cortisol response to stress than the group-housed fish. It is suggested that the lower anxiety and cortisol in isolated fish may be because a lack of feedback from conspecifics dampens the stress response (Parker et al. 2012; Giacomini et al. 2015).

It is unclear why the expected effect of increased anxiety after social isolation was seen in some studies but not others. The strain of zebrafish used can affect the outcomes of behavioural tests (Lima et al. 2016), but, of the five studies mentioned above, three used wild-type short fin fish (Parker et al. 2012; Pagnussat et al. 2013; Giacomini et al. 2015), while the other two used the AB strain, which was derived from wild-type fish (Collymore et al. 2015; Shams et al. 2015), and so the anxiety response appears unrelated to strain in this situation. The range of effects reported indicates that there is much to be learned about how zebrafish are affected by social isolation; there is a paucity of data on its effects in different settings and situations (Parker et al. 2012; Collymore et al. 2015), and so more research is required before the specifics of different responses can be clarified. The existing studies have generally investigated overall mean effects of isolation, not whether individuals with prior levels of anxiety react to social isolation in different ways. Thus, the findings from the social isolation chapter of my thesis, while not set up for pairwise analysis of individuals before and after treatment, are a valuable addition to the literature.

There is great potential for future research along the lines of this study to provide further insight into the consistency of behaviours across contexts. If I were to do the social isolation experiment again, I would run an initial anxiety test to give the fish experience in the tank and with being handled, and then conduct a second test a week later to phenotype them properly. I would also do the experiment with tagged focal individuals, allowing me to assess the individual repeatability of the behaviour across context. I would use larger group sizes to reduce the confounding effect of unstable dominance hierarchies in a small group (Pavlidis et al. 2013; Rudin et al. 2016). A further extension of this experiment could be to return the isolated fish to group housing after the test period, and determine whether they would return to baseline anxiety levels. This could help clarify whether the changes in behaviour I saw were temporary reactions to stress, or whether the changes were longer-lasting. It would be fascinating to determine how stressful a situation must be for a generally repeatable behaviour to change within individuals, and whether this threshold might differ between categories of behaviour. This could be better done with tagged individuals, to enable more direct comparisons of behaviours before and after treatment, and also with larger, more appropriate group sizes for the group-housed individuals. It would also be interesting to expose the fish to the test tanks once before the initial phenotyping, to see whether some of the changes observed in this study were because of different initial behaviour causing misidentification of anxious and non-anxious individuals. Understanding whether changes in anxious behaviour after a challenge are underpinned by alterations in gene expression could provide insight into the link between phenotypic changes and gene expression. While delays with sequencing precluded gene expression data from being included in this thesis, I plan to use the samples collected from the fish used in this experiment to explore this link in the future.

Much research is done on animal behaviours, but, of that, not many researchers study the repeatability of those behaviours. Additionally, individual variation is often neglected in favour of studying the group means, which erases valuable information about individual personalities (Royauté et al. 2015). We now know that this individual variation in behaviour, despite previously being thought of as random non-adaptive variation around an adaptive mean, shows different strategies for survival and reproduction (Réale et al. 2007), and may have important consequences for conservation and captive breeding (McDougall et al. 2006). Translocation programs are increasingly taking personality into account when individuals are chosen for transfer, because there is evidence that selection of individuals with a range of personality types can enhance translocation success (Watters and Meehan 2007; Powell and Gartner 2011).

### 4.3 Conclusions

The major findings from this study were that activity-, anxiety-, and exploration-related (and, to a lesser degree, boldness-related) behaviours were repeatable over a six-month period, and that social isolation is enough of a behavioural challenge to alter prior anxiety levels, especially in initially non-anxious fish. I also found evidence of both sex differences and behavioural syndromes over time. These findings contribute to the established literature by giving an idea of how consistent some behaviours are over time and after a change in context. It provides long-term behavioural repeatability estimates, which have not previously been widely studied or reported in the literature. Additionally, the thesis findings give an idea of how a change in social context (that is, social isolation) alters the anxiety-related behaviour of zebrafish which have been previously categorised as anxious or non-anxious. I have also identified potential areas of interest for further studies into zebrafish personality and social behaviour.

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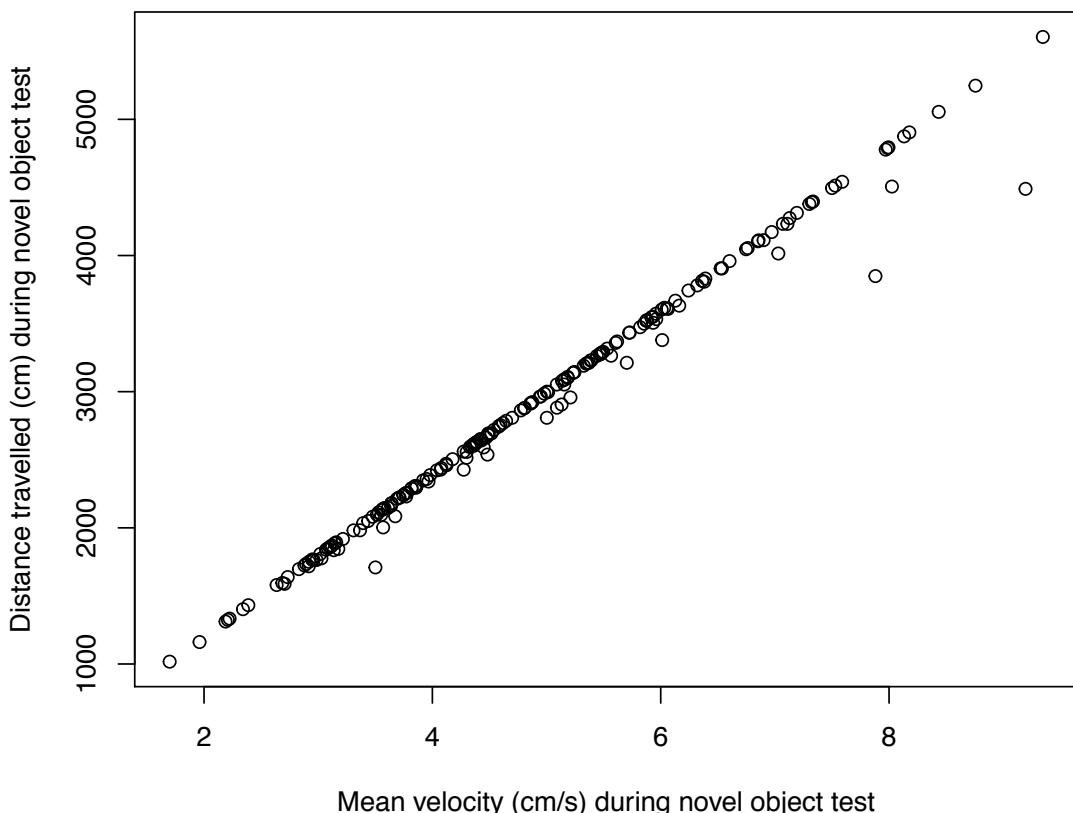
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# Appendices

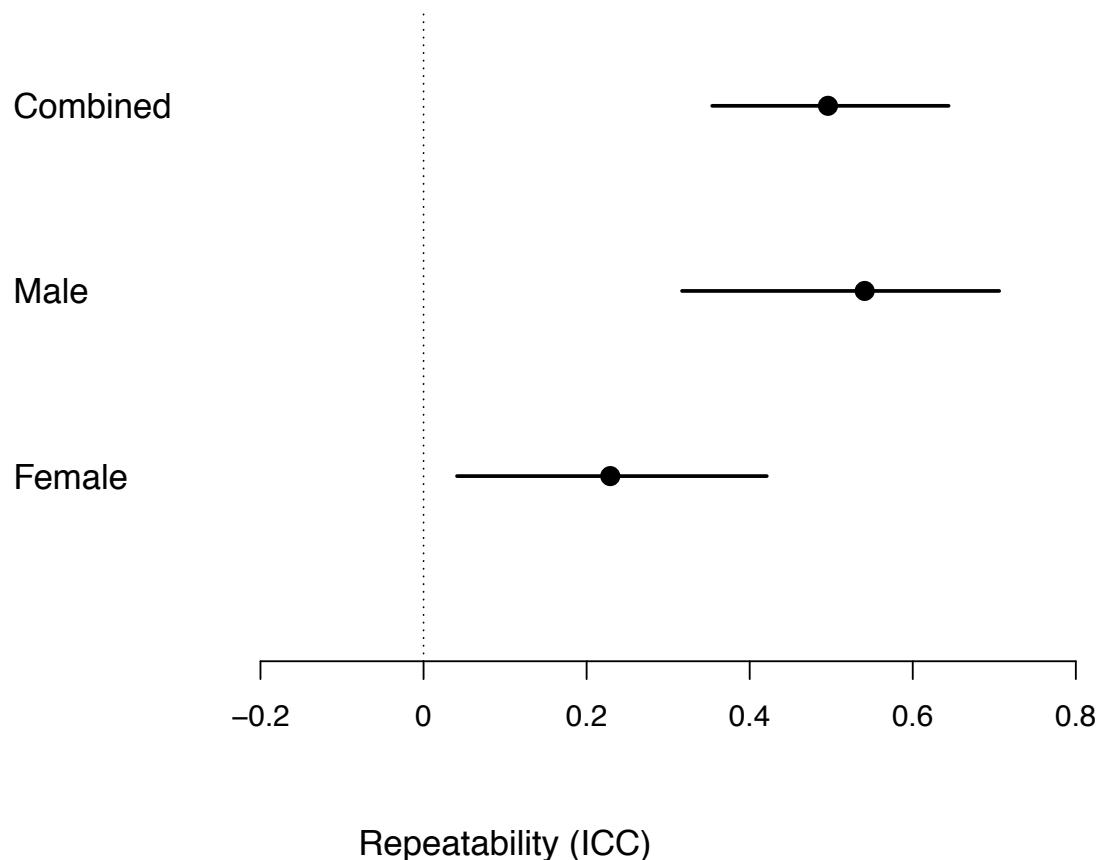
## Appendix 1

Distance travelled was highly correlated with mean velocity, as shown using a linear regression (adjusted  $R^2 = 0.987$ ,  $p < 0.001$ ; Fig. A1). Thus, velocity was not included in the main text to avoid repetition.



*Figure A1:* Correlation between distance travelled (in cm) and mean velocity (in cm/s) over all five time points studied. Total n = 205 (41 individuals with 5 repeats each).

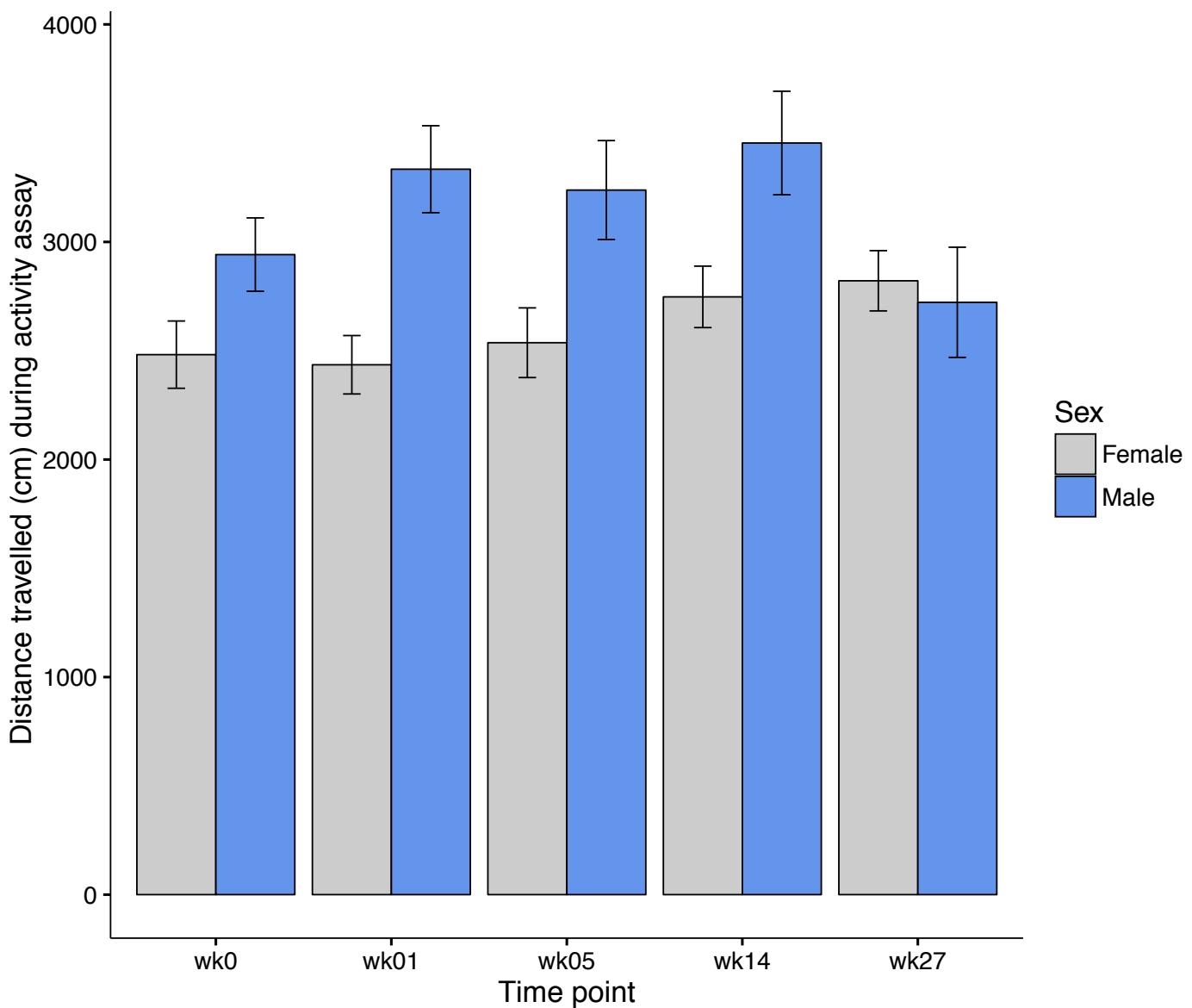
Mean velocity was significantly repeatable overall ( $R= 0.496$ ; 95% CI= 0.354, 0.644). Males were more repeatable ( $R= 0.541$ ; 95% CI= 0.317, 0.706) than females ( $R= 0.229$ ; 95% CI= 0.041, 0.421). The repeatability values for velocity (Fig. A2) are very similar to those for distance travelled (Fig. 2.3.1).



*Figure A2:* Repeatability estimates and 95% confidence intervals for mean velocity over all time points. Estimates are shown for both sexes combined, as well as males and females separately.  $n = 21$  males and 20 females,  $k = 5$  repeats.

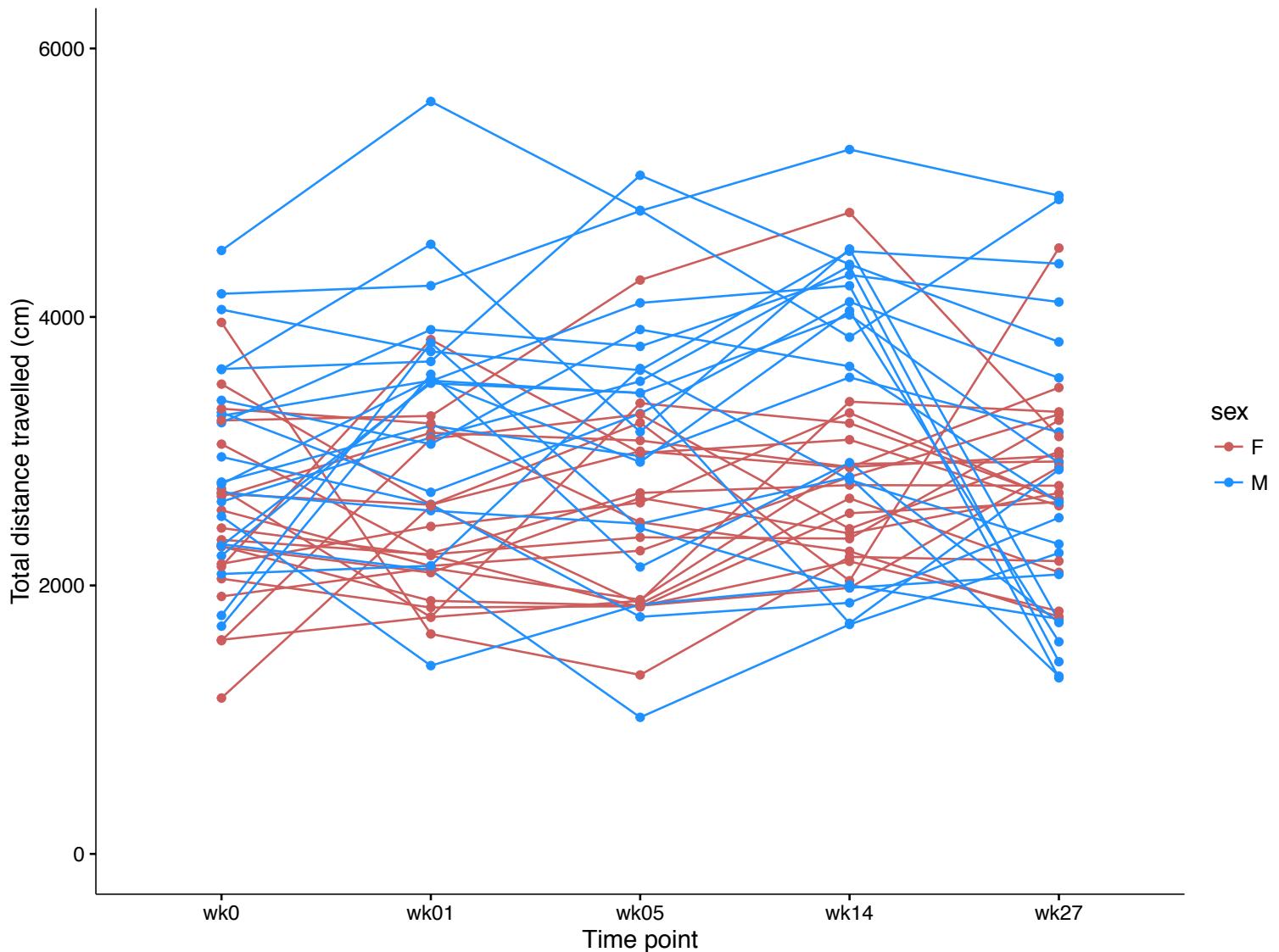
## Appendix 2

Males consistently travelled farther than females except in the 27 week test. The mean distance travelled also changed more over time in males than in females, despite higher individual repeatability in males (Fig. A3).



*Figure A3:* Mean distance travelled ( $\pm$  standard error) during the activity assay by males (blue) and females (grey) at each of the five time points tested. n = 20 females and 21 males.

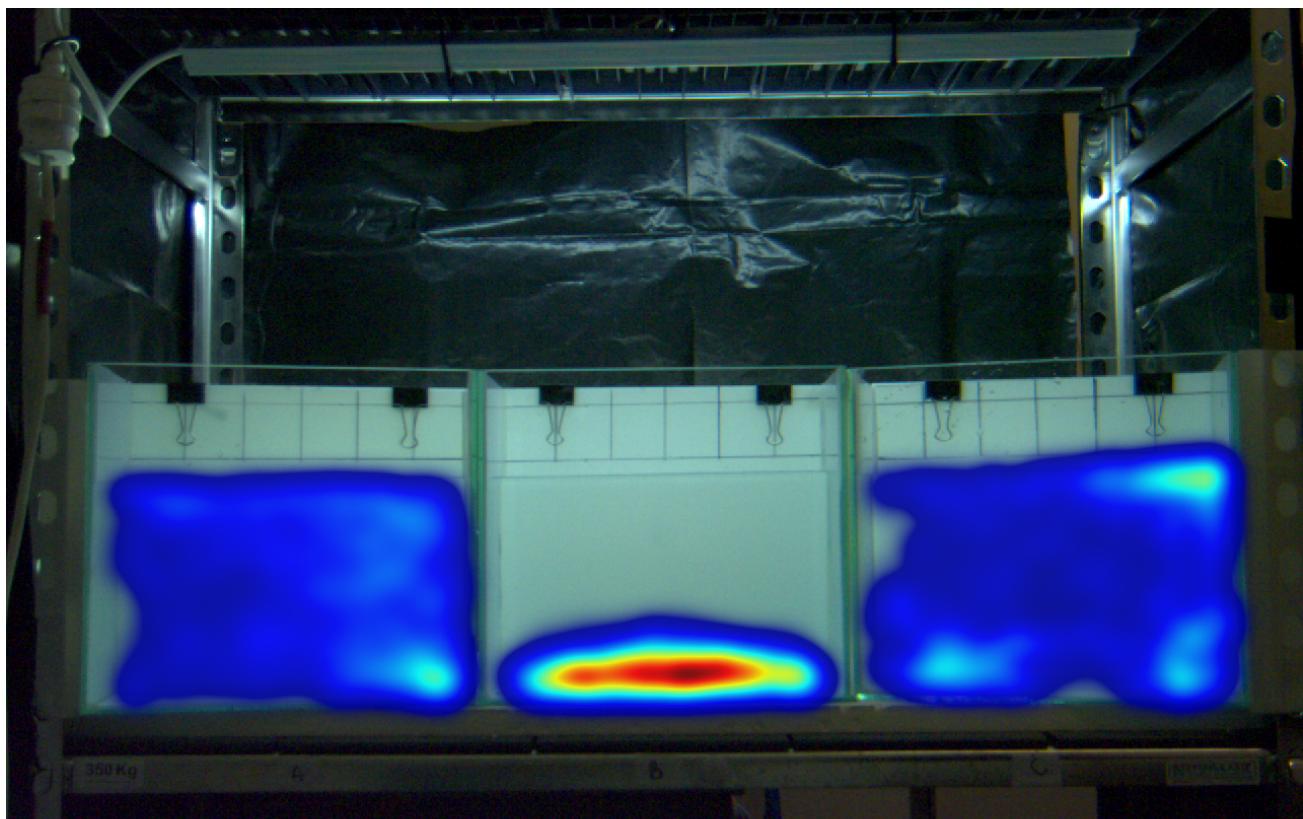
Some individuals showed different changes in distance travelled over time than the mean group-level changes. Individual changes in behaviour are shown as a reaction norm plot below (Fig. A4).



*Figure A4:* Reaction norm plot showing changes in distance travelled during the novel arena test over the five time points by males (blue) and females (red). Each line shows one individual. n = 20 females and 21 males.

### Appendix 3

Exploration for an individual was calculated using the sum of the standard deviations of the cumulative duration in seconds spent in each of the nine zones during the novel arena test. Thus, if a fish explores all zones equally, it is exploratory, and has a low standard deviation. Conversely, if a fish stays in just a few zones for the whole test, it is not exploratory, and has a high standard deviation. To confirm that this is an accurate measure of exploratory behaviour, I compared the standard deviation values with heat maps of the trials. For example, the heat map below shows the exploratory behaviour of three fish (Fig. A5). The fish in the leftmost tank was exploratory, and had a standard deviation of 33.1s; the fish in the rightmost tank was similarly exploratory, and had a standard deviation of 39.9s. The fish in the middle tank, however, was not highly exploratory, and had a standard deviation of 111s.



*Figure A5:* Exploratory behaviour of three fish visualised using heat maps. The two individuals in the outside tanks were exploratory, while the fish in the middle tank was not exploratory.

## Appendix 4

The ratio of individuals that did approach the novel object to those that did not approach changed over time; very few individuals approached the novel object during the first three time points, while 50% of individuals did approach it at weeks 14 and 27 (Fig. A6).

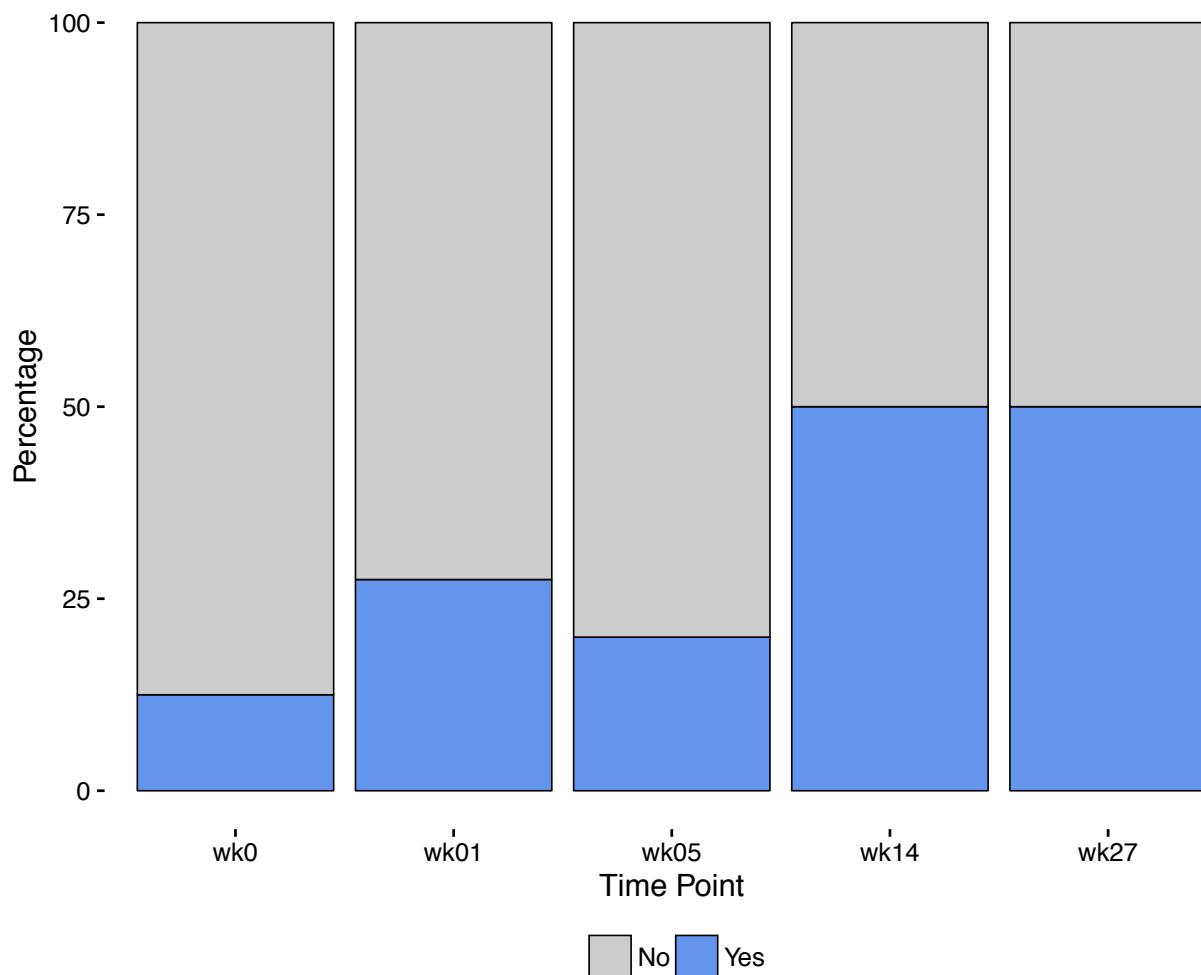
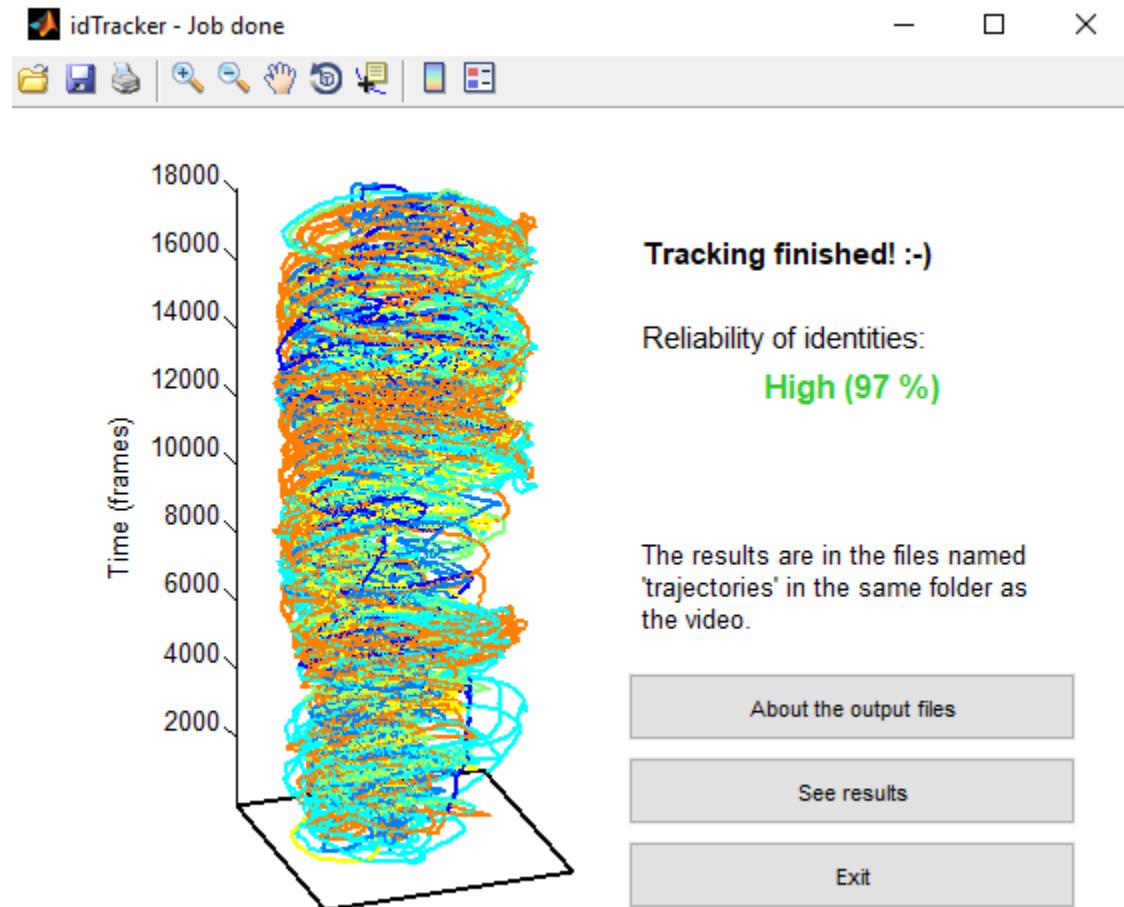


Figure A6: The percent of individuals which approached (yes) or didn't approach (no) the novel object during the boldness trial over the five time points tested. n = 40 at each time point.

## Appendix 5

The tracking success of the sociability videos analysed using idTracker was high for every one of the sociability videos (Fig. A7).



*Figure A7:* The window which shows reliability of identities after idTracker completes a video analysis. This image is from a sociability test with six individuals. The graph shows the position of all individuals in the test arena (horizontal axes) at each frame of the video (vertical axis).

The R code below was developed in our lab to analyse idTracker output from videos with multiple fish in order to obtain estimates for sociability. The code also calculates total distance travelled and mean velocity, as well as latency to cross a boundary. Behaviours for two time periods within the same video footage can be calculated.

```
# Script to get mean nearest neighbour distance (MNND; i.e. group cohesion), nearest  
neighbour distance (NND), distance travelled, average speed, space-use and latency time  
from raw data from IDtracker
```

```
library(TSdist)  
library(dplyr)  
library(ggplot2)  
library(reshape2)  
library(proxy)  
library(plyr)  
library(adehabitat)  
library(adehabitatLT)  
  
rm(list=ls(all=TRUE))  
data<-read.table("27weeks_t4_males.txt", h=T)  
head(data)  
  
#some little manipulations to get each row assigned with their respective frame reference  
l<-length(data[,1])  
fra<-as.vector(seq(1,l,1))  
data<-cbind(data, fra)  
head(data)  
  
#filtering data by probabilities using the filter function from dplyr:  
prob<-0.5 #the level of confidence you want  
data_f<-filter(data, ProbId1 > prob, ProbId2 > prob, ProbId2 > prob, ProbId3 > prob,  
ProbId4 > prob, ProbId5 > prob, ProbId6 > prob)  
  
#extract length of this new, trimmed, dataset and get time steps  
l2<-length(data_f[,1])  
time<-seq(1,l2,1)
```

```

# extract the reference of the remaining frames after trimming (to monitor gaps after
trimming)
fra2<-data_f$fra

#Create a data frame where coordinates (X, Y) of each fish are grouped by their time step.
This part is a bit heavy, and could surely be coded more elegantly...but anyway, it works. If
you are dealing with a different number of fish just adapt the code accordingly by adding or
removing IDs

ID1<-cbind(data_f$X1, data_f$Y1, rep(1,l2), time)
ID2<-cbind(data_f$X2,data_f$Y2, rep(2,l2), time)
ID3<-cbind(data_f$X3, data_f$Y3, rep(3,l2),time)
ID4<-cbind(data_f$X4, data_f$Y4, rep(4,l2), time)
ID5<-cbind(data_f$X5, data_f$Y5, rep(5,l2), time)
ID6<-cbind(data_f$X6, data_f$Y6, rep(6,l2), time)

matrix<-as.matrix(rbind(ID1, ID2, ID3, ID4, ID5, ID6))
colnames(matrix)<-paste(c("X", "Y", "ID", "time"), sep = "")
head(matrix)
coord.list<-as.data.frame(matrix)
head(coord.list)
s<-split(coord.list, time)

#check that you have the coordinates of the 6 fish at time step#1
s[[1]]

#two functions computing the mean neighbour distance (MNND) and nearest neighbour
distance (NND) from the distance matrix computed at each time step.

MNND_F<-function(x){round(colMeans.dist(dist(x), diag = F))}

#returns, for each frame, the average distance for each column of the distance matrix (i.e.
each individual), excluding the diagonal of 0's

```

```
NND_F<-function(x){apply(as.matrix(dist(x)), 1, FUN = function(x) {min(x[x > 0])})}  
#returns, for each frame, the minimum distance for each column of the distance matrix,  
outside the diagonal
```

```
#apply the function the entire data frame (and transpose it as well)  
MNND_t<-t(sapply(s, NND_F))  
NND_t<-t(sapply(s, NND_F))
```

```
#merge the MNND and NND output with its associated frame reference (in order to visualise  
when data were cropped). Distances are in pixels, but you would just need to multiply with  
which ever conversion coefficient you have from filming a scale.
```

```
MNND<-as.data.frame(cbind(MNND_t, fra2))  
colnames(MNND)<-paste(c("Id1", "Id2", "Id3", "Id4", "Id5", "Id6", "frame"), sep="")  
head(MNND)
```

```
#average of the MNND:  
MNND_av<-colMeans(MNND[,1:6])
```

```
#if you want to compare average inter-fish distances between 2 time periods, before and after  
a stimuli at 120s (3600 frame)  
stimuli<-3600  
MNND_av1<-colMeans(filter(MNND, frame<stimuli))[1:6]  
MNND_av2<-colMeans(filter(MNND, frame>stimuli))[1:6]
```

```
NND<-as.data.frame(cbind(NND_t, fra2))  
colnames(NND)<-paste(c("Id1", "Id2", "Id3", "Id4", "Id5", "frame"), sep="")  
#average of the NND  
NND_av<-colMeans(NND[,1:6])  
NND_av1<-colMeans(filter(NND, frame<stimuli))[1:6]  
NND_av2<-colMeans(filter(NND, frame>stimuli))[1:6]
```

```
# graph MNND and NND per frame for each fish
```

```

md<-melt(MNND, id.vars = "frame")
ggplot(md, aes(frame,value, col=variable)) + geom_line()

d<-melt(NND, id.vars = "frame")
ggplot(d, aes(frame,value, col=variable)) + geom_line()

# you can then look at variation patterns, look at specific fish, or just visualise change in the
shoaling behaviour though time.

##### speed, distance and space-use

# for speed, we need some time reference, in our case, it's the frame ref. We need to convert
frame into time, so we need to specify the frame rate of our video (usually 30fps but need to
be adapted if filmed in slow-motion)

FPS<-30

# convert frames into time

s2<-as.data.frame(cbind(matrix, fra2/FPS))

#include it in a new general data frame

colnames(s2)<-paste(c("x", "y", "id", "order", "tms"), sep = "")

head(s2) #just to check how the dataset looks

## function to extract coordinates every n frames - in order to compute speed on larger
spatio-temporal scale - in our case positions are recorded 30 times per second (once per
frame), we could then extract one position every 30 recordings, to get a distance in pixels/
seconds that you can convert later on into cm/s

n.extract<-function(dataframe, n)dataframe[seq(n,to=nrow(dataframe),by=n),]

s2<-n.extract(s2, 30)

head(s2)

ta<-as.vector(s2["tms"])

#get time standardised to create ltraj object from our coordinates (you can't have two records
per time stamp, hence the need to get one position per second)
time= as.POSIXct(as.character(Sys.time()), tz = "GMT") + as.numeric(unlist(ta))

```

```

locs<-as.data.frame(cbind(s2,time))

#check

head(locs)

#create ltraj object

traject<-as.ltraj(xy=locs[, c("x","y")], date=locs$time, id=locs$id, typeII=TRUE)
summary(traject)

# just some checks to see some specificities of the ltraj object and how to browse through it
head(traject[[1]])

plot(traject)

hist(traject[1], "dist", freq=TRUE)

#total distance travelled (in pixels)

tot_dist<-c(1:5)

for (i in 1:5){

  a<-sum(traject[[i]]$dist, na.rm=TRUE)

  tot_dist[i]<-a

}

tot_dist

#if you want to compare distance travelled between parts of the video, before and after
stimulation (same as before, but we change frames into seconds: 120)

stim<-120

s21<-filter(s2, tms<stim)

ta1<-as.vector(s21["tms"])

head(s21)

time1= as.POSIXct(as.character(Sys.time()), tz = "GMT") + as.numeric(unlist(ta1))

locs1<-as.data.frame(cbind(s21,time1))

traject1<-as.ltraj(xy=locs1[, c("x","y")], date=locs1$time1, id=locs1$id, typeII=TRUE)
summary(traject1)

#total distance travelled (in pixels) for period 1

```

```

tot_dist1<-c(1:6)
for (i in 1:6){
  a<-sum(traject1[[i]]$dist, na.rm=TRUE)
  tot_dist1[i]<-a
}
tot_dist1

#total distance travelled (in pixels) for period 2
s22<-filter(s2, tms>stim)
ta2<-as.vector(s22["tms"])
head(s22)
time2= as.POSIXct(as.character(Sys.time()), tz = "GMT") + as.numeric(unlist(ta2))
locs2<-as.data.frame(cbind(s22,time2))
traject2<-as.ltraj(xy=locs2[, c("x","y")], date=locs2$time2, id=locs2$id, typeII=TRUE)
summary(traject2)

#total distance travelled (in pixels) for periode 1
tot_dist2<-c(1:6)
for (i in 1:6){
  a<-sum(traject2[[i]]$dist, na.rm=TRUE)
  tot_dist2[i]<-a
}
tot_dist2

# average speed through moving time window (in this case 10 seconds).
av_speed <- sliwinltr(traject, function(x) mean(x$dist/x$dt, na.rm = TRUE), step = 10, type =
"time", units = "s")
#extract speed data for fish 1, just as an example
av_speed[[1]]

#average speed over the entire recording based on previous computation with moving mean
(if you want it based on each time step, just set step=1)
global_av_speed<-c(1:6)
for (i in 1:6){

```

```

global_av_speed[i]<-mean(av_speed[[i]]$y, na.rm=TRUE)
}

#average speed for the 6 fish
global_av_speed

#same thing but for the two periods defined previously:
#Before stimuli (first 2 min):
# average speed through moving time window (in this case 10 seconds).
av_speed1 <- sliwinltr(traject1, function(x) mean(x$dist/x$dt, na.rm = TRUE), step = 10,
type = "time", units = "s")
#extract speed data for fish 1, just as an example
av_speed1[[1]]

#average speed over the whole of period 1
global_av_speed1<-c(1:6)
for (i in 1:6){
  global_av_speed1[i]<-mean(av_speed1[[i]]$y, na.rm=TRUE)
}
#average speed for the 6 fish for period 1
global_av_speed1

#After stimuli:
av_speed2 <- sliwinltr(traject2, function(x) mean(x$dist/x$dt, na.rm = TRUE), step = 10,
type = "time", units = "s")
#extract speed data for fish 1, just as an example
av_speed2[[1]]

#average speed over the whole of period 2
global_av_speed2<-c(1:6)
for (i in 1:6){
  global_av_speed2[i]<-mean(av_speed2[[i]]$y, na.rm=TRUE)
}

```

```

#average speed for the 6 fish for period 2
global_av_speed2

##### kernel density analysis just for descriptive analysis of space-use
xy<-locs[, c("x","y")]
id <- locs$id
ud<-kernelUD(xy, locs$id, h = "href", grid = 50)
udvol <- getvolumeUD(ud)
image(udvol, zlim = c(0, 90))

#gen stat without period comparison:
gen_stat<-cbind(MNND_av, NND_av, tot_dist, global_av_speed)
gen_stat #distances are in pixels

```