# The Influence of Active and Passive Sampling Techniques on the Sampling of Bold and Shy Zebrafish in a Laboratory Based Study

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**Abstract**

One of the most cited behavioural type indices in animal studies is the bold and shy index. Bolder animals are observed to be more exploratory, have a higher propensity to take risks, and are more dominant than their shyer counterparts. These bold and shy behavioural types are varied between individuals amongst a population; therefore, allowing for a diverse range of these behavioural types. All studies aiming to address a specific issue on a population have to sample from the population. The hope is that the sample will represent the diversity and individual variation seen in the population. This is done using an essence of randomization in the sampling. However, these behavioural types can affect different types of sampling techniques — overrepresenting and creating systematic trapping bias. This study examined the idea that passive sampling methods may sample bolder animals, and that animals that are consistently bolder and more exploratory will be more likely to be sampled by methods that require voluntary inspection of the trap. This study addressed the possible oversampling of bolder zebrafish by passive sampling methods in a laboratory setting by sampling animals from a home tank using two active sampling methods and two passive sampling methods. All the fish sampled were tested using the Novel Tank Test to test anxiety and exploratory behaviour, and the Emergence Test to test for boldness. Thus, allowing for an assessment of the possible sampling bias caused by passive sampling methods as well as a test for behavioural correlates. There was no significant difference in the occurrence of bold vs shy fish number trapped by either passive or sampling methods. Thus, in this paradigm, each of the sampling methods sampled a diverse range of both bold and shy behavioural traits.

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## 1. Introduction

Zebrafish have been emerging as a prominent animal model for translational behavioural neuroscience. This emergence is due in large to their homology to humans. However, as this animal model grows, so do possible confounding variables that are not sufficiently addressed. Zebrafish, similar to many different animals, have been observed to exhibit consistent behavioural temperaments that are persistent through temporal and situational contexts. These individual variations or "personalities" influence many different aspects when it comes to research, such as the ability of the research to make inferences from a sample to a population. For an investigation to have this predictive power, there needs to be randomization when it comes to sampling the population. However, if the persistent and constant behavioural temperaments influence these sampling methods, which had been observed in limited studies, then the inferential capabilities of the study are heavily confounded. Therefore, if thought and consideration are not put into the sampling procedures, then those procedures may over-represent a specific behavioural type.

### 1.1 Zebrafish in Behavioural Neuroscience

Throughout different areas of scientific research, the use of zebrafish as a model has been gaining tremendous popularity. The use of Zebrafish in developmental research has been popular because of their nearly transparent embryos and rapid developmental rate. Their use in genetic research has gained popularity because of their similar genetic structure to humans, external fertilization, high fecundity, rapid development and high stocking densities, which allow them to be genetically tractable (Lieschke & Currie, 2007; Kalueff et al. 2013). Thus, zebrafish have been cemented as a useful model for research towards vertebrate development and genetic diseases such as muscular dystrophy and cancer. The next and ongoing pursuit is to use zebrafish to investigate areas of behavioural neuroscience.

Within behavioural neuroscience, the predominant models of human condition are rats and mice. These models have been highly effective in helping investigate animal cognition, learning, memory, and other behavioural functions. In order to investigate these behavioural functions, there have been extensive experiments and behavioural assays created. The Radial arm maze to test memory, the open field test to measure anxiety, and the emergence test to measure exploratory behaviour and boldness, to name a few prominent examples. The reason why these models are predominately used is because they fit the facets that are required for an animal model; A good model needs to demonstrate face-validity (the model looks like it measures the condition in question), construct validity (whether it measures what it claims to measure) and predictive power to make those generalizable inferences to the population (the overall predictive ability) (Gould & Gottesman, 2006).

For an animal model to be effective, it needs to be able to represent a particular human condition. In order to model that particular condition, an animal model should demonstrate some degree of homology to humans. This homology can come in different aspects, such as their physiology, anatomy, genetic composition, and behavior (Kalueff et al. 2013). There has been a plethora of research that has echoed these homologous properties for zebrafish. As mentioned, they have significant genetic homology to humans. A study by Barbazuk et al.

(2000) mapped positions for 523 genes and expressed sequence tags. Eighty percent of the zebrafish and human genes analyzed belonged to conserved synteny groups (two or more genes linked in both humans and zebrafish). Allowing for considerable ability to map and investigate genetic diseases. Similarly, through large-scale forward-genetic screens, genetic mutations within zebrafish have been recovered that are orthologous to human diseases, showing apparent phenotypic similarities (Lieschke & Currie, 2007).

Zebrafish and humans also share fundamental anatomical brain areas; Anatomical areas including fore-, mid- and hindbrain, peripheral nervous system, specialized sensory organs (e.g., visual, olfactory, and auditory system), and stress response system. With these brain areas, the zebrafish is also able to exhibit higher order behaviours and integrated neural function homologs to humans. These include memory, conditioned responses and social behaviours (Lieschke & Currie, 2007). Further, the involvement of the amygdala and habenula in zebrafish affective behaviours parallels human data on these structures (Kalueff et al 2014). This is further supported by the research that shows that zebrafish possess all major neuro mediators systems, including neurotransmitter receptors, transporters, and enzymes of synthesis and metabolism, similar to those observed in humans and rodents (Kalueff et al. 2014) — allowing for the considerable ability to investigate morphological issues as well as fundamental high-order behavioural issues, such as issues with memory and learning.

The Zebrafish CNS has high physiological homology to humans, and because of this homology, different studies have been conducted to investigate aspects of brain disorders and diseases (Kaleuf et al. 2013; Stewart et al. 2014). When looking at brain imaging, it was found that there are 53 defined structures at an isotropic resolution of 10 µm, making it the highest resolution magnetic resonance (MR) atlas in any species examined thus far. The 53 defined brain structures are within the major brain divisions; Telencephalon, Diencephalon, Mesencephalon, Rhombencephalon, Brain Stem, Spinal tracts, commissures, and ventricles. And, the structures are within the minor brain divisions, which are Praeoptica, epithalamus, thalamus, posterior tuberculum, hypothalamus, pretectum, textum opticum, torus semicurcularis, tegmentum, cerebellum, and the medulla oblongata. This resolution of these brain structures is essential for imaging regions and their substructures in the zebrafish brain. It can assess both the CNS morphological abnormalities (e.g., occurring as a result of a disease) and the gross morphology of various brain regions and their connectivity (Ullmann et al. 2010: Stewart et al. 2014). Similarly, these brain areas and any damage to them from disease or brain disorder can also be studied.

To tie it all together, one study by Saif et al. (2013) stated that zebrafish are gaining popularity in behavioural neuroscience research as this species combines practical simplicity with system complexity. The practical simplicity in this statement is the ease of use. In comparison to other animal models, zebrafish are practically simple; their behaviours are more efficiently measure, rapid and external development of their transparent embryos, the ease of reproduction, and their transient lifespan. All these factors allow them to have a sense of practicality in neuroscience research in comparison to mammalian models. The systems' complexity in this statement refers to the principles that define a useful animal model. The animal model can effectively model a condition in humans and show some degree of homology. As mentioned, zebrafish show this homology with high internal complexity and similarity based on the comparisons mentioned earlier. (Brustein et al. 2003; Howe et al. 2013). This system's homology helps the zebrafish model translate back to people to understand a human condition further. As referenced by many studies, the zebrafish is commonly called a complimentary model or an alternative model that benefits only alongside mammalian models (Levin et al. 2007; Kari et al. 2007; Kalueff et al. 2014) However, as time progresses and different studies continue to research zebrafish, the model may even take precedence over a mammalian one.

### 1.1.1 Zebrafish Model Comparison to Mammalian Models

One of the most common mammals used in translational research is the rat. Paradigms developed for the rat has influenced behavioural tests and paradigms used for zebrafish research. Tests such as the T maze test, the open-field test, and the emergence test are all adapted from the rat paradigm, and these tests are thought to measure analogous behavioural processes seen in both the rat and zebrafish. For example, the T-maze tests working memory for fish as it does in rats or mices, the open field test (which is the novel tank test for zebrafish) measures the level of anxiety, and the emergence test measures the animal's exploratory behaviour and boldness (Rosemberg et al. 2011; Stewart et al. 2014) .

Another example of the adaptation from a mammalian model to the zebrafish model is the Chronic Unpredictable Stress Paradigm. The rat model for depression research uses this chronic unpredictable stress paradigm (CUS), which characterized by inducing chronic stressors onto the rat, and then assessing their subsequent behaviours and endocrine responses (Bondi et al. 2008; Chakravaty et al. 2013; Piato et al. 2011). These chronic stressors are induced to mimic the chronic stresses faced by humans and how that stress has been shown to manifest different cognitive deficits and psychological conditions, such as major depressive disorder. The chronic stressors that are induced to mammalian model cause the animal to develop regimented learned behaviours and can be a list of many things, such as restraint, crowding, isolation, novelty, temperature, and any form of dangerous stimuli. (Kaleuf et al. 2014; Bondi et al. 2007; Piato et al. 2011). Studies have been using CUS extensively because the paradigm elicits anxiety-like behaviours, cognitive deficits, and subsequent drug use that systematically mimic depression and anxiety that studies observe in humans (Haile et al. 2001; Bondi et al. 2007). With this, behavioural tests can assess novel pharmaceutical interventions towards a variety of psychological disorders by measuring the behaviours exhibited by the rat after the drug's induction. They showed this very concept in a study by Bondi et al. (2007), which showed decreased anxiety in animals exposed to CUS after antidepressant medicine induction in the elevated plus-maze.

Comparatively, Zebrafish research uses the same paradigm when investigating depression and anxiety. The research also echoes the same results when the studies use similar chronic stressors. This is because zebrafish share similar stress response system projections. In humans and mammals this stress response goes through the Hypothalamic-Pituitary-Adrenal (HPA) Axis, and for zebrafish and other teleost this stress response is facilitated by the Hyopthalamic-Pituitary-Interrenal (HPI) axis (Egna et al. 2009). They also induce the fish with similar type stressor such as isolation, novelty, danger mimicking stimuli (i.e., an image of a predator or a constructed replica), tank cooling, lower water levels, and alarm pheromone stress. They then observe the fish's robust behaviour and relate one animal model with another (Chakravarty et al. 2013; Piato et al. 2011).

Zebrafish research, like mammalian research, measures higher-order cognitive aspects, such as learning and memory. Zebrafish can be tested in different formulated assays that measure the animal's spatial memory, as demonstrated in a study by Williams et al. (2002). They used a simple spatial alternation paradigm for food reward where the fish were fed on alternating sides of a divided fish tank, with a red card displayed on one side serving as a cue for the reward (i.e. the food). They found that the fish learned that the red card indicated the availability of food, and thus would swim towards it whenever they saw the red card. Zebrafish have also been used in tests of non-associative learning. It is corresponding to habituation of the startle response in larval zebrafish and the habituation to novelty in different novel stimuli tests (Wolman et al. 2011; Wong et al. 2010). Zebrafish have been tested in classical conditioning procedures, the most popular being the conditioned place preference. In this paradigm, a stimulus (i.e. a drug) is paired with a specific context, and preference for that specific context is subsequently measured. That context is the conditioned stimulus, and is usually based around the visual system and colour or area of a tank (Webb et al. 2009; Blaser &Vera. 2014). Zebrafish have also been used in operant conditioning procedures, in which consequence or reward is contingent on the behaviour of the organisms. These procedures could be based around food or aversion and usually implanted within some variation of a shuttle box, T-maze, plus maze, and using either simple response discrimination or visual cues. Intuitively, these processes are more difficult to induce in zebrafish and require more training trials, and they also have a higher degree of confounding variables. However, the research has been done and continues to expand – opening the avenue for further research (Blaser & Vera. 2014).

Among the more common assays in research adapted from mammalian models is the test of anxiety and habituation, the Novel Tank Test (NTT) and the Emergence Test(ET). NTT is similar to the open field test in rats or any CUS tests that focus on the animal's anxiety levels when entering a novel area (Stewart et al. 2010). Similarly, ET for fish species mimics the ET in rats where the animal goes from an area of refuge to an open and riskier area, in which the performance is affected by the animal's boldness and exploratory inhibitions.

These different tests measure different types of conditions and, thus, different behaviour outputs. In some research, these behaviours outputs measured in tests have been consistent in other tests measuring different conditions (Riechert & Hedrick. 1993; Huntingford. 1976; Michelangeli et al. 2016). These behaviour seem to be constant from temporal, situational, and environmental changes (Toms, Echevarria, & Jouandot. 2010). Thus, an animal that is exploratory and active in the Emergence Test would then, theoretically, be less anxious in the Novel Tank Test and would explore more. This suggests a constant underlying tendency that influences behavior in a consistent manner across similar situations. This consistency of behaviours is observed prevalently in humans. Someone shy in one context would be more likely to be shy in another, and those consistent behavioural patterns in humans are referred to as personality traits (Trillmich & Hudson. 2011; Gosling. 2008; Stamps. 2007) . However, this consistent innate and individual behaviours, through subsequent experiments in animals, have given rise to a variety of research questions that spark further investigation around the individual differences in fish. This generalized consistency of behaviour in individuals suggest that the fish may have intrinsic and individual personalities.

### 1.2 Personality research in Zebrafish

Even though there are limitations when using an animal model to look at "personality" research, many studies still investigate this anthropomorphic innate representation. In a simple sense, it is impossible to look at any research without addressing the idea of "personality" in animals. "Personality" is one aspect that differentiates and individualizes every single person in a population, causing a variation in individuals within a species. This individual variation is arguably one of the critical drivers of evolution and natural progress because of aspects like trade-offs and adaption (Stamps. 2007; Nettle. 2006; Dingemanse & Reale. 2005; Bergmuller & Taborsky. 2010; Stamps & Groothuis. 2010). For humans, this term is spread around without hesitation.

Consequently, attributing "personality" to animals has been on the minds of researchers. However, unlike studying people, there is the worry that studying personality in animals would be regarded as a soft science and approach the "scientific sin' of anthropomorphism. The argument was that attributing humanistic qualities to animals devalues the validity of the research and makes it heavily subjective (Ogden. 2012; Eddy et al. 1993; Horowitz & Bekoff. 2007). However, animal "personality" is still relevant in biological science research. The difference is that studies mask the term "personality" with different vernacular, words such as behavioural syndromes, coping styles, animal temperament, and inter-individual variation. And, with those terms, comes objective operational definitions that are able to be measured. These terms are all synonymous with the “personality” of the animal that this present study will be discussing (Ogden. 2012; Biro &Stamps, 2008; Koolhaas et al.

1999; Sih et al. 2004; Groothius & Carere. 2005; Koolhas et al. 1999; Bergmuller & Taborsky. 2010).

Throughout the literature, researchers agree that within-species, individuals vary consistently with their response to environmental stimuli and social environment stimuli, whether that be shared social environment or non-shared social environments (Bergmuller & Taborsky. 2010). And consequently, this variation will affect the organism's overall productivity and ability to survive (Bergmuller &Taborsky. 2010; Biro &Stamps, 2008) These variations are persistent through a variety of different environmental and temporal contexts and have cluster of adjacent correlated behaviours that occur together. These "personalities" and correlated behaviours have been well documented in many vertebrates, including salamanders/skinks (Sih, Kats & Maurer, 2003; Michelangeli et al. 2015), fish (Wilson et al. 2011; Overli et al. 2005), birds (Groothuis & Carere. 2005; Garamszegi et al. 2009), rodents (Koolhaas *et al.*, 1999) and other mammals [e.g. mink (Malmkvist & Hansen, 2002), bighorn sheep (Reale *et al.*, 2000)].

In a meta-analysis study, Sih et al. (2004) state that: "A behavioural syndrome is a suite of correlated behaviours expressed either within a given behavioural context or across different contexts”. For example, some individuals (and phenotypes) might be more aggressive, active, and "bold," while others are less aggressive, less active, and “shy”. The correlation between behaviours has a lot to do with fitness, the ability to survive to reproductive age, find a mate and produce offspring (Sih. 1980; Sih et al. 2004; Biro & Stamps. 2008). The more offspring an organism produces during its lifetime, the higher it's biological fitness. Any behaviour that challenges that fitness will display a negative correlation. For example, if the animal spends too much time looking for food and not enough time avoiding predation, those two behaviours will end up being negatively correlated. Therefore, there is a need to balance the two conflicting demands (Sih. 1980). This has been discussed in terms of domain-general and domain-specific behavioural traits, where domain-general refers to an absolute consistency in behaviour in different times and situations, and domain-specific refers to the behaviour being dependent on the context (i.e., the situation and time). The literature supports both hypotheses, suggesting that behavioural traits are between the spectrum of domain-general and domain-specific (Reale et al. 2000; SIh et al. 2004). Similarly, the development of consistent behavioural phenotypes and correlated behaviour has also been attributed to the specialization of social niches through the interactions of the immediate shared social environment and the non-shared social environment through life development (Bergmuller & Taborsky. 2010). In other words, the organisms diversify their personality slightly from other conspecifics based on physical environmental stimuli and intrinsic needs to alleviate conflict and progress their evolutionary fitness.  For instance, a bolder animal will be more exploratory, and more dominant based on their development in their environment, which will diversify their social niche from other members of the population. That social niche will not overlap/conflict with those animals that are shyer, reserved, and less exploratory.

Behavioural traits that carry over across contexts show behavioural correlations. An example of this would be different settings and tests where the animal exhibits similar aggressive and bold behaviours. In studies with zebrafish and spiders, territorial aggression (agonistic behavior) was positively correlated with boldness towards predators (anti-predator behavior), meaning that when the zebrafish and spider acted quicker towards predators they would subsequentially be more aggressive when claiming territory against their conspecifics.

(Riechert & Hedrick. 1993; Huntingford. 1976). Similarly, in a study by Michelangeli et al. 2016, they found a behaviour syndrome in skinks, which showed positive correlations between activity, exploratory behaviour, and sociability. They sampled skinks (*Lampropholis delicata*), a species of terrestrial lizards, using three different sampling methods. They used an active sampling method of hand capture, a passive sampling method of pitfall traps (pitfall traps are containers recessed into the ground and are used to capture terrestrial animals; they fall into the traps and cannot get out) and another passive sampling method of mealworm fishing (involved the use of a wooden pole with some fishing line attached, a sinker, and a piece of cotton thread was attached to the fishing line, and a mealworm). They conducted a series of behavioural assays to examine the variation and correlation of five common behavioural traits: activity, exploration, sociability, foraging activity, and boldness. They also aimed to assess whether the sampling methods would oversample a particular behavioural type because with these correlated behaviours, comes the question of sampling bias and increased attention to specific sampling methodologies. As mentioned, they found behavioural correlates, and with these correlations, they observed that bolder skinks would show more locomotor activity, explore areas that shyer individuals may not, and be friendlier to the individual's conspecifics. But, they found no difference in the behavioural types caught by the three trapping methods. However, even though they found no differences, it continues to raise the question of a possible sampling bias caused by correlated behaviours.

The study of "personality" in animals is, in itself, fascinating. However, from an experimental consideration, this consistent response style could be a confounding variable that will be affected by the sampling method used and impact the behavioural assays conducted.

The foundation of experimental design is based on the random collection of subjects for the assignment of different levels of the dependent variable. Random selection implies that all individuals in the subject group have an equal chance of being selected. However., By its very nature, an animal with bold or shy characteristics may vary the number of subjects in the subject group selected depending on the method of selection (Michelangeli et al. 2015; Stuber et al. 2013). For example, when netting fish from an aquarium for random assignment, the animals that are swimming along the top of the tank may be inherently bolder, active, and more exploratory and therefore, would influence the subsequent behavioural tests. Similarly, using a passive sampling method like a trap box that stays immobile in the area where the organisms reside would bias the sample. The very nature of a passive method revolves around the need for voluntary and active choice by the animal, which completely isolates individuals who are consistently inactive, reserved, or shy (Biro. 2013). Therefore, it is crucial for the interpretation that the design of experiments consider and understand this behavioural variation between individuals and its temporal and contextual consistency.

Behavioural traits such as the bold/shy domain have been well documented in many different animals, including fish. In a study by Wilson et al. (1993) involving juvenile pumpkinseed sunfish, they implemented tests, and those tests were designed to measure the response to a novel object (latency to approach) to test for behavioural trait of boldness (WIlson et al. 1993 as referenced by Gosling. 2001). They had found that, a pumpkin seed fish that was “bolder” when approaching the novel object also showed signs of boldness in different areas: likely to approach a human observer and quicker to acclimatize to a newer environment. This consistency is present because of the inherent characteristic within the definition of “personality,” especially when observing it through an evolutionary and ecological perspective. In a study by Harcourt et al. (2009) using three-spine sticklebacks (*Gasterosteus aculeatus*), the fish were identified as bold or shy by an assessment that investigates “boldness” concerning risk aversion. Intuitively, the bolder fish would have a higher propensity to take risks, and the opposite seen for shy fish. This risk aversion test involved the fish exiting an area of safety to get food in an open space that posed a more significant risk to the fish. They performed a median split and then conducted a preference test. This preference was focused on the categorical variable of the bold and shy label to see how they would differ in preference test for different types of shoals, whether the shoals primarily filled with bold fish or shy fish would be chosen. The results found that both types of fish would associate themselves more with bolder shoals and that a bolder fish when introduced to either bold or shy shoal, would increase that shoal’s activity. With that finding, shoals, ideally, should be varied when it comes to the personality traits of the fish that are within it. However, within the shoals, bold individuals are still more likely to explore out to areas that are more risky/dangerous. This study also revealed that bold shoals have higher foraging success than shy shoals. Still, when the fish were food-deprived, the bold fish were competitors to the shy fish, instead of mutual benefiters (Dyer et al. 2008: Magurran. 1990 as referenced by Harcourt et al. 2009). These studies show that shoaling behaviour can also occur in a laboratory setting and that they are influenced by consistent behavioural types, which should then be impacted by different sampling techniques and consequently effect different behavioural tests.

### 1.2.1Bold/Shy Index

Even though boldness is one of the most assessed traits in non-human personality research, there are still discrepancies in how different studies operationally define the term boldness. One of the reasons is because boldness is an overarching term for many conjoined and correlated behaviours. For example, some studies define bold type animals as those that react actively to threatening situations by addressing the situation quickly. Conversely, shy type animals act carefully and prefer to wait passively for a threat to pass (Dahlbom et al. 2011), which showcases boldness as risk-taking behaviour. However, risk-taking behaviour can incapsulate different behaviours as well. In a study by Dahlbom et al. (2011), they explored if boldness could be used to predict social status in zebrafish. To begin, they assessed boldness, and they did so by monitoring individual zebrafish behaviour in three different assays. These assays were an unfamiliar barren environment with no shelter (open field), an unfamiliar environment with the addition of a shelter where the animal resides, and a familiar environment with a foreign object (Lego® brick) presented. The assessment for boldness for all three tests had an aspect of novelty and risk. The study then assesses the dominance level (either dominant or subordinate) of each fish through a dyadic tournament to investigate the relationship between boldness and dominance, a correlated behaviour. The study goes on to show that behaviours expressed by the fish during the boldness tests could be used to predict which would later become dominant and subordinate in the tournament—concluding that bold behaviour is positively correlated to dominance. However, in this particular instance, the focus is the specific assessment of boldness, which in this case, is the aspect of novelty in the open field, emergence, and novel object test.

As mentioned earlier, another common assessment for boldness relates to the tendency of the animal to take more risks, which a multitude of assay can assess (Reale et al. 2007). Dahlbom et al. (2011) shows this with his study. Alternatively, in other papers, the definition refers more to the ease of capture of the animal. In this sense, the bolder animal will openly investigate a trap (Sih et al. 2004; Wilson et al. 1993; Michelangeli et al. 2015). However, there is still striking similarity to the ease of capture and risk-taking behaviour. In a study by Stuber et al. (2013), they assessed the exploratory behaviour of the great tit bird and assessed its correlation with boldness and locomotor activity. In this study, they used nest boxes that the animal roosts inside to sample the individuals. Afterwards, they varied the nest box by adding a novel object (a camera) within it. This variation was implemented to see if the tit bird would continue to roost in the nest box. The hypothesis was that a bolder animal would be more likely to roost in a nest box that has a novel object inside it. After all the individuals were sampled, they then ran behavioural assays for boldness and exploratory behavior using a cage with different areas for the bird to explore, and they found a positive correlation. They theorized that when an animal is measured to be bolder, it will consequently explore more, and thus, the animal will move more. This entire study showcases a blend of both an aspect of novelty, risk-taking and the ease of capture to assess boldness.

The novelty and exploratory risk-taking are common elements of boldness assessments. However, some researchers believe that boldness and shyness are not related to novelty but are related solely to the propensity of an animal to take risks (Reale et al. 2007). In this study by Reale et al. (2007), they divide common behavioural traits into five categories: shyness-boldness, exploration-avoidance, activity, sociability and aggressiveness. The boldness-shyness index is defined and measured as an individual's reaction to any risky situation, but not any novel situation, which differs from most studies assessing boldness. The definition that Real et al. (2007) posits includes a response to threatening conditions, such as predators and humans. However, the study does not omit novelty completely; instead, the association of novelty is in the definition of the behavioural traits: exploration and avoidance. This exclusion of novelty has been argued by other scientists and researchers, who defend the inclusion of a novel aspect as a determinant for boldness, and they claim that boldness is overarching exploration/avoidance, risk-taking, activity, and many other traits.

The majority of the shy/bold index assays involve a varied response to novelty (i.e. NTT, novel object test, etc.) (Dalhbom et al. 2011; Coleman and Wilson, 1996; Egan et al. 2009: Wong et al. .2010; Egan et al. 2009; Maximino et al. 2010; Reider & Connaughton, 2015; Boon et al. 2008; Burns. 2008). This inconsistency of the determinants of a personality trait is in part because fish personality research is relatively new and that researching one single personality trait, like boldness or shyness, is very rudimentary and does not consider the comorbidity of behavioural traits. As mentioned earlier, Reale et al. (2007) argued this when they excluded the idea of novelty in their study of boldness and shyness and suggesting that the novelty has more to do with exploratory/avoidance. This association is not entirely wrong in consideration with the literation. After the introduction of  correlated behaviour, It has been understood and assessed that when an animal exhibits one specific trait (ex. boldness), then that animal is also more likely to exhibit another trait that is positively correlated (ex. boldness and exploratory behaviour) (Sih et al. 2004).

### 1.2.2 Other Behavioural Traits

With various studies showing that different behaviours can positively correlate to one another, it is only appropriate to address those other behaviour types that are related to the bold/shy continuum. The behaviours that have been studied to correlate with bold/shy are exploration, aggression, and locomotor activity (Sih et al., .2004; Riechert & Hedrick. 1993; Huntingford. 1976; Dingemanse et al. 2002; Michelangeli et al. 2015; Stuber et al. 2013)

The literature defines aggression as any negative behaviour (agonistic) exhibited by the animal. Examples of those behaviours would be biting or any physical force towards other fishes, conspecifics or not (Larson et al. 2006; Norton & Balley-Cuif. 2010; Way et al. 2015; Dahlbom et al 2011). When measuring this trait, researchers use many different tests, such as a mirror in the tank or having another fish in the tank but separated by a partition (Marks et al. 2005). An act of aggression relates to boldness because a more aggressive fish is more likely to take risks in any situation that poses a threat (Sih et al., .2004; Riechert & Hedrick. 1993; Huntingford. 1976; Ariyomo & Watt. 2012)

Exploratory behaviour is also positively correlated with boldness. There is much debate about how to operationally define and measure how explorative an animal is. Most studies that look at exploration measure it using an aspect of novelty, which effectively, makes it similar to boldness (Dingemanse et al. 2002; Reale et al. 2007; Red'ko et al. 2015; Boon et al .2008; Burns. 2008). This aspect may be a novel area, object, or even a new stimulus subject. The amount of time spent on the novel object, around the novel stimulus subject, or spent exploring novel areas would be used as the dependent variable to measure exploration. That exploratory behaviour observed is seen to be positively correlated with boldness behaviour because those new areas that the animal explores could pose a possible risk (Dingemanse et al. 2007).

The third behavioural trait mentioned is locomotor activity. Boldness and exploratory behaviour are both positively correlated with locomotor activity. Animals that explore more are undeniably more active than those that do not. For example, it was shown that bolder skinks explored more and therefore had higher activity scores than those skinks that were shy (Michelangeli et al. 2015), and the exploratory behaviour of the great tit bird positively correlated with boldness and activity (Stuber et al. 2013). These last two studies focus on an aspect that is heavily confounded by the behaviours/personality of the animal, which is if there is a relatively passive trap, it would seem those traps would catch bold animals, thus oversampling those animals (Michelangeli et al. 2015: Stuber et al. 2013; Garamszegi et al. 2009). This could impact any inferences taken from that study that generalize behavioural traits due to the over-representation of bold animals in the sample.

### 1.3 Sampling in Research and Random Selection

This oversampling poses a problem with the validity of a study. Studies that involve understanding any issue relevant to a population needs to include random selection, and the random selection relies on random sampling or the process by which each element in a population has an equal chance of selection (O'leary. 2004). This process eliminates researcher bias and allows for statistical estimations of representativeness, and It is the only choice for researchers wishing to do advanced statistical analysis (O'leary. 2004). Random selection of the sample also allows for a higher amount of predictive validity. With a more diverse sample, there can be a generalization to the entire population. However, if the sample does not have a distribution of every individual, the validity of the study would be in question. Oversampling of animals with a specific behavioural trait may affect not only the validity of behavioural studies but also any research done that examines physiological, behavioural aspects, and life-history traits. (Biro & Stamps 2008).

Animal studies have highlighted this by showing how Passive Sampling Methods which rely on the animal to inspect and intentionally arrive at the trap, oversample bold animals. (Michelangeli et al. 2015; Stuber et al. 2013; Biro. 2013). Therefore, an individual's behaviour type has been seen to influence the probability of that animal to be caught and sampled (Michelangeli et al. 2015; Stuber et al. 2013; Garamszegi et al. 2009). In contrast to Passive Sampling Methods, Active Sampling Methods do not rely on the animal approaching and inspecting the trap. Instead, the researchers, in charge of the sampling, use methods where the involvement of the animal's choice is limited (Michelangeli et al. 2015; Wilson et al. 2011). This turns to the question that this current study was designed to address, whether specific sampling techniques tend to oversample specific behavioural types, specifically passive sampling methods oversampling bold type animals.

### 1.3.1 Passive and Active Sampling Methods

Sampling methods typically fall into two categories: passive versus active dimensions. Some examples of passive sampling methods, which rely on the intentional investigation, are funnel/bottle traps, nest traps, mealworm traps, baited traps, pitfall trap, and mist-netting (Michelangeli et al., 2015; Garamszengi et al. 2009; Stuber et al. .2013). Some examples of more active methods, which do not require the intentional investigation, are gillnets, hoop nets, hand trapping, hand netting, seines, and most other netting procedures (Port et al. 2006; Michelangeli et al. 2015; Stuber et al. 2013). This intentional choice of the animal to investigate passive sampling methods is why, theoretically, bolder animals should oversampled when passive sampling methods are only used.

**1.4 Present Study**

The present study was designed to determine whether active or passive sampling techniques would tend to oversample fish of either bold or shy temperament (Toms, Echevarria, & Jouandot, 2010: Riechert & Hedrick. 1993; Huntingford. 1976; Sih et al. 2004). While much has been studied about the bold/shy index and the correlated behaviour associated with it, there remains a need to further the biases that those behaviour types cause on sampling when different sampling methods are used. Currently, there is very little research the biases that sampling methods have on collecting different behavioural types. Which propelled this present study to examine this possible sampling bias, and whether passive methods produced an oversampling of bold type fish. Zebrafish were sampled from a home tank using the two different sampling methods: Active and Passive. The hypothesis was that the passive methods would oversample bolder fish because of methods requirement for voluntary inspection. Consequently, the active methods would then sample a diverse range of both behavioural types. These methods were then further divided into four branching methods: two Passive Sampling Methods and two Active Sampling Methods. The two Passive Sampling Methods were variations of the bottle trap (used for individually sampling fish), and the two Active Sampling Methods were two different hand netting procedures.

The Zebrafish were also tested in two different behavioural assays: The Emergence Test (ET) and the Novel Tank Test (NTT). The ET is often used to measure the boldness and explorablity of a fish. It is also used in labeling a fish as either “bold” or “shy” through a median split, which is dependent on the sample’s median latency to emerge. This use of the ET mimics the use in this present study; the sample of Zebrafish will be labelled either bold or shy based on the median split of the ET results. Before the ET, the Zebrafish were tested in the NTT on an array of measures to attempt to replicate the results of correlated behaviours that the literature has observed.

The findings of the study attempted to exemplify the importance of addressing specific sampling methodologies and how they relate to the bias of the oversampled behavioural type. This study focused on the effect of sampling bias on predictive and concurrent validity of a study, delving into the importance of a genuinely randomized sample.

## 1.5 Behavioural Assays

### 1.5.1 Novel Tank Test

A widely used experiment to test the boldness and exploratory behaviour of fish is the NTT. The NTT focuses on the natural tendency of zebrafish to initially dive to the bottom of a new experimental tank, with a gradual increase in vertical activity over time as the fish habituates to its new environment(Egan et al., 2009; Reider & Connaughton, 2015; Blaser & Rosemburg, 2012; Burns. 2008). The longer the fish spends at the bottom of the tank is a representation of the fish's anxiety level, which comes with being exposed to a novel area (Blaser & Rosemburg, 2012). In other words, it exploits the fish's instinctual and natural tendency to seek protection in an unfamiliar environment. The one way the fish does this, if there are not any overt areas to hide, is to remain at the lower end of the tank (or any novel area) until they feel safe enough to explore (Egan et al. 2009). Novel tanks vary in depth for different studies. However, it usually is divided into two or three or four horizontal sections outlined by a line or quadrants on the outside of the aquarium. (Egan et al. 2009; Rosemburg et al. 2011). The dimensions of the experimental aquarium and the natural tendency of the fish allow researchers to measure the latency to leave the bottom section of the tank and how often the fish moves from one section of the tank to the other. This test measures the level of anxiety of the fish as well as its activity level, with the assumption that bolder fish will be less anxious and more exploratory than shyer fish.

### 1.5.2 Emergence Test

Another widely used behavioural assay to measure boldness is the ET. The ET focuses on the animal's response to a novel area as well. However, with this test, the fish is initially placed in a small enclosure and held for a limited period. This enclosure is used as a safe refuge place for the fish and it initially gets acclimatized to this area. Following this, a door is raised, allowing the fish to leave the enclosure and enter the novel area. The latency of the fish to emerge to a new area is measured; The time it takes for the animal to emerge from the sheltered location to the novel environment is the dependent measure (White et al. .2013; Burns. 2008). The latency to emerge is related to how bold the animal is because bolder animals, theoretically, are not as anxious when it comes to being introduced to a new environment and, thus, should emerge sooner (Brown & Braithwaite. 2004).

## 2 Methods

### 2.1 Subjects

Forty zebrafished were used in this study. All the zebrafish (n=40) were originally obtained from the commercial supplier One Fish Two Fish Red Fish Blue Fish (Charlottetown, PE) from a home tank of approximately 210. The fish were sampled from a house 20 gal tank, and then quarantined in a 10 gal tank after testing. The water temperature in each tank was kept at approximately 25° C. The fish were fed daily (TetraMin® Tropical Flakes) for five-minute intervals before and during test days. All fish were mainated on a 12:12 light/dark cycle (on at 7 am and off at 8 pm). Water for filtration was provided by the Atlantic Veterinary College and quality tests were run on the water daily for nitrate, nitrite, and ammonia. All 40 fish remained in the home tank prior to sampling and in the quarantine housing tank after.

### 2.2 Equipment/Sampling Methods

These methods were decided on by the level of invasiveness and voluntary movement/choice of the zebrafish. The first sampling method was an Active Sampling Method. It was a netting procedure characterized by causing the least amount of stress onto the fish residing in the home tank. The method sampled fish from the top 15 cm of the tank and collected those that were easiest to catch, which disturbed the fewest fish. The second method was also an Active Sampling Method, but this netting procedure was characterized by choosing the fish deeper in the tank, namely the bottom 15cm, and focusing less on the ease of capture. Both netting procedures were done with a 33cm hand net.

The remaining two sampling methods were both Passive Methods, which used bottle traps. The bottle traps required the intentional choice of the fish, highlighting the distinction between Passive and Active Sampling Methods. The bottle traps were constructed using plastic bottles (502.8ml) with the top opening cut off and placed upside down, similar to a funnel (14cm tall and 7cm wide). Small holes were drilled near the top, and fishing twine was put through those holes to allow for the bottle traps to be lifted. Holes were also drilled on the side and sanded so that the holes were smooth; this was done so that the fish would not be harmed by any rough edges entering or exiting the trap. The holes also allowed for water to exit the bottle. Thus, when the traps were lifted, water would escape, but enough would remain still to allow the fish to remain immobile but comfortable within the bottle (see Figure 1). For the passive methods, the fish intentionally entered the trap and then were taken out individually. The first passive sampling bottle trap was empty, but the second passive sampling bottle trap had a small novel red lego brick inside it. For both Passive and Active Sampling Methods, after the fish were sampled, they were then immediately tested in the behavioural assays, starting with the NTT and followed by the ET.

**2.3 Procedure**

Within the population of zebrafish, 40 were selected from the population in the home tank of 210 fish; 10 using each sampling method n=40. The study began with the use of Active Sampling Methods; Both Active Sampling Methods used hand nets to select the fish, and random order for the Active Sampling Methods was created using a random number generator. Once a fish was sampled, it went directly into the behavioural tests. Fish were individually run through behaviour testing. These experiments were the Novel Tank Test (NTT), and the Emergence Test (ET) (see Figures 2 and 3). All fish were tested through the behavioural assays in this order: First in the NTT, and then the ET. The Active Sampling Methods took three days to complete. The Passive Sampling Methods started on day three, immediately following the end of the Active Sampling Methods. There were four bottle traps, two for each passive sampling method. All four bottles were placed in the home tank at once, and they were taken out as the fish entered them. Once a fish entered the trap, the trap was removed, and the fish then individually assed in the behavioural assays mentioned, in the same order and succession.Then, that trap was returned to the home tank

Each of the 40 fish were tested in the NTT. They were placed individually in the novel tank. The NTTs has 40x21x23cm dimensions (see Figure 3). Behind the tank was a white presentation board, and on the tank were three parallel lines dividing the tank into thirds, which is illustrated by horizontal lines on the outside of the tank. A light was placed above the area of experimentation, and one outside the experimentation room; those lights were the only lights on during experimentation (48 lux +/- (5% + 2 d)). The camera was placed in the front of the tank. The individuals were taken according to a specific sampling method and then placed in the center of the test tank. They were then allowed to swim around the tank freely for a time of 6-minutes. The following behaviours recorded: latency (s) to reach the upper third portion of the tank, time (s) spent in the bottom third, time (s) spent in the middle third, time (s) spent in the top third, number of transitions made between segments (entries), and freezing. Freezing was characterized as a total absence of movement, except for the gills and eyes, for a one second or longer.

Following the NTT, the fish were placed in the ET. ET tank was 40x21x23cm (see Figure 2). The refuge area for the fish was created using a PVC tube with two openings with one other end closed off using aquarium sealant and a plastic cover. Fishing twine was attached to the plastic cap so that it could easily be opened for the fish to leave the refuge area. A piece of black mesh was placed on the top so that it gave the refuge a dark environment. The camera was put in front of the tank, facing and focusing on the opening of the refuge area. Once the fish were secure in the refuge area, they were given two minutes to acclimatize. After that, the plastic door was opened, and the latency (s) of the fish to leave the refuge area was measured. The fish would leave the refuge area once the whole-body length left the area.

### 2.4 Data Analysis

All behavioural trials were video recorded for later evaluation. For both the ET and the NTT, the videos were analyzed visually using the BORIS event logging/coding software. The primary measures for the NT included the latency (s) to initially cross the first line of the bottom third, number of crosses past the first line of the bottom third, number of crosses past the second line of the middle third, total crosses, total time (s) spent in the bottom third, total time (s) spent in the middle third, total time (s) spent in the top third, total time (s) immobile.

For the ET, the primary measure is the latency to emerge from the refuge area. This measurement was chosen in order to divide the sample of fish into a bold and shy category. The other primary measures were chosen to address the different correlated behaviour traits that construct the bold behavior syndromes and temperaments.

Statistical analyses were performed using SPSS 25 (IBM Statistics, Canada), and graphs were generated in Microsoft Excel. A one-way ANOVA with a posthoc test (Bonferroni) was performed to evaluate the effect of sampling methods on behavioural measures in the emergence tank and NTT. A median split was performed on the latency to emerge in the emergence tank to objectively label bold and shy zebrafish. The shy fish were those that fell above the median, and the bold fish were those that fell below. Another one-way ANOVA was performed to evaluate the effect of boldness on the behavioural measures in the ET and the NTT to see if they correctly conformed with the literature cited on the bold/shy index. A Chi-Square test was performed to test the relationship between the two categorical variables: The Sampling Methods and the bold/shy label.

**3.Results**

Adult Zebrafish were tested in the NTT and the Emergence from four different sampling methods. One-way ANOVA revealed no statistically significant difference between the sampling methods on each of the behavioural measures in the NTT and ET (See Table 1).

A median split was performed on the latency to emerge dependent variable that was recorded in the ET. This median split divided the sample of fish into two defining categories. A fish would fall into the shy category if their emergence time fell above the median, which would mean they were slower than the median times. A fish would fall into the other category bold if their emergence time fell below the median, which would mean they were quicker than the median time to emerge. Once this was completed, the sample of 40 fish were divided into bold (n=20) and shy(n=20). This allowed for further Analysis of variance between this categorical variable in the dependent measures. It also allowed for a Chi-square analysis to be performed between the two variables: the sampling method and the behavioural traits. The Chi-square analysis test revealed no significant relationship between sampling methods and the bold or shy fish. $X^2(3) = .80$, $p > .05$. (see Table 2)

Mean +/- S.E.M of each dependent measure as a function of sampling type and behavioural type were also obtained (see Table 3 and Table 4)

Another One-way ANOVA was performed on the effect of boldness on the dependent measures saw a significant difference in each of the dependent measures against boldness. Against boldness, using the fish that were labelled bold and shy through the median split of emergence time in the ET, there were significant differences seen in the total number of crosses, where the bold labelled fish made significantly more crosses than the shy $F(1,38) = 16.463$, $p<.001$. A significant difference in time spent in the bottom third, where the bold fish spent significantly less time in the bottom third than shy fish $F(1,38) = 23.789$, $p<.001$. A significant difference in time spent in the middle third, where bold fish spent significantly more time in the middle third than shy fish $F(1,38) = 15.576$, $p<.001$. A significant difference in time spent in the top third were bold fish spent significantly more time in the top third than shy fish $F(1,38) = 17.386$, $p<.001$. A significant difference in time spent immobile, where the bold fish spent significantly less time immobile than the shy fish $F(1,38) = 12.232$, $p<.001$. Furthermore, a significant difference in latency to make a first cross, where the bold fish had a significantly quicker latency to make the first cross than shy fish $F(1,38) = 27.210$, $p<.001$. Bar graphs were created of the significant values (see Figures 4 through 10)

**4. Discussion**

This research investigation aimed to evaluate the effect of sampling techniques on the collection of bold and shy zebrafish. The research accomplished this by observing well-characterized behavioural measures in the NTT and ET while using four different sampling methods. These sampling methods were two hand netting procedures, and two bottle traps, which were under two overarching ways of sampling; active and passive. The results indicated that there was no significant effect on boldness and shyness against the sampling methods, which accepts the null hypothesis of an evenly distributed sample of behavioural traits (bold/shy) amongst the four sampling methods. There was also no significant difference in sampling methods against each of the dependent measures.

There has been little research on the effect of the bold/shy behavioural traits on sampling methods in adult Zebrafish, with even little research on laboratory-housed adult zebrafish. In the most comparable investigations on the effect of behavioural traits on sampling bias, the researchers focused on other animals. Studies by Michelangeli et al. (2015), Stuber et al. (2013), Carter et al. (2012), and Biro, P. A. (2013), point out the importance of sampling bias and how the oversampling of animals with one type of correlated behaviours would affect the study. In the investigations by Stuber et al. (2013), Carter et al. (2012), and Biro, P. A. (2013), they used passive traps (nest boxes, claptraps, and gillnetting, respectively). They found that more active and faster-exploring individuals (tit birds, rock agamas, and rainbow trout, respectively) would enter the passive traps more than the slower exploring individuals. The study by Michelangeli et al. (2015) did not find the same type of correlation. They used three sampling methods; one active hand capture method and two passive methods (mealworm fishing and pit traps) with delicate skink. They found no difference between the individuals caught in the three trapping methods among the five behavioural traits they measured. In the present study, all the sampling methods collected an even distribution of bold and shy fish.

This fueled the investigation into sampling bias amongst zebrafish. The bold and shy fish were classified using the ET, which is a test of risk-taking and exploration (Harcourt et al. 2009: Naslund et al. .2015). Once the fish were labelled (bold or shy label), An ANOVA test was conducted for the bold and shy labels against the different behavioural measures. This analysis saw a significant difference in each of the measures. Suggesting that a bolder fish was also more active and had a higher number of total crosses than a shyer fish. This finding supported the literature on behaviours that are positively correlated to boldness (exploratory and locomotion).

However, there was no difference seen between the individuals and the sampling methods used to catch them; all sampling methods sampled a diverse range of individuals. The hand netting procedures sampling a diverse range of bold and shy fish echoes most studies using active methods. These methods do not involve voluntary interaction, and the animals do not have to inspect or approach the trap. So, theoretically, a bolder animal that is more risk-taking, more active, and exploratory does not pose an inherent bias on Active Sampling Methods. However, the bottle traps also sampled a diverse range of bold and shy fish. These results go against the hypothesis that Passive Sampling Methods would sample bolder animals, which studies that used Passive Sampling Methods observed (Stuber et al. 2013: Carter et al.

2012: Biro, P. A. 2013). One study that does mimic the passive sampling finding was done by Michelangeli et al. (2015). During their investigation, the pitfall trap and the mealworm fishing methods used, which are both somewhat passive methods, collected a wide variety of bold and shy behavioural type delicate skinks. They theorized that trapping bias is not ubiquitous and may only be associated with those passive traps that involve the response of animals to novelty. One issue that pertains to their methodology is that their passive methods do not adequately fall under the definition of passive sampling. The pitfall traps did not allow the animal to investigate the trap voluntarily. These traps were unbaited and hidden, and thus, they would fall closer to active sampling methods. The mealworm fishing was done close to sheltered areas where the delicate skinks reside, which allow for shyer individuals to feel comfortable enough to investigate the bait. With these considerations, it is relevant to notice possible confounding variables that would allow the sampling techniques to collect a diverse range of bold and shy fish. Even though the bottle traps used in the present study were in a familiar area for the zebrafish (their hometank), the traps required investigation from the zebrafish. If these bold and shy individuals display behaviours that are distinct from temporal, situational, and environmental changes, then it should be noticed that the bolder animals would investigate the passive traps more often regardless of the safe and familiar context (Toms, Echevarria, & Jouandot, 2010: Stuber et al. 2013: Carter et al. 2012: Biro, P. A. 2013). One possible reason for this result could be due to the animals, in the literature cited, being wild-reared animals. As discussed before, animals can still be varied in their behavioural temperaments within a laboratory setting (Alvarez & Nicieza. 2003). However, the expressions of these behaviours could slightly differ between wild-type and lab animals. During the animal's formative years, their development is heavily influenced by their environmental situation, and these situations greatly vary between wild- and lab-reared animals. This reason would also support domain-specific behavioural type literature, in which the context that the animals fall influences the exhibited behavioural output.

There have been many studies that showed differences in laboratory-reared animals and wild-type (Berejikian. 1996: Wright et al. 2006; Alvarez & Nicieza. 2003; Blanchard et al. 1986; Burns et al. 2009; Mineka et al. 1980). One way that this difference is shown is through the way zebrafish behave in shoals. Boldness, in shoaling behaviour, has been seen to be decreased in domesticated zebrafish, which could be because of the fixed density, abundant food, and no predation observed in laboratory settings. Therefore, there is no inherent need for fish to congregate into shoals for foraging reasons and no need for the animal to be bolder. Similarly, a study by Alverez & Nicieza (2003) found the same behavioural differences when it came to antipredator behaviour between wild and hatchery-reared brown trout. The hatchery-reared and wild trout differed in their response to predators where the offspring of wild fish reared under hatchery conditions did not react to the presence of predators. They hypothesized that this divergence could be due to the hatchery-reared fish’s lack of experience with predator or predation instances, especially during early developmental years, where the need to avoid predators establishes. This would imply that the fish can learn to recognize and avoid predators at a young age and is not a predisposed behaviour. This difference in anti-predatory behaviour between wild-type and laboratory-reared animals has been seen in other studies as well (Alvarez & Nicieza. 2003; Nettle. 2006; Sih et al. 2004; BErekikian et al. 1996). Returning back to the adult zebrafish used in this present study, these fish lived in the laboratory tank for extended periods. As learning and habituation proceed, the fish become conditioned within the laboratory environment. There is no intrinsic need for the animal to be bolder when it is within a large group, and the resources are abundant. (Wright et al.2006: Wright et al. 2003: Way et al. 2015).

There is ample food for them on a routine schedule, and the fish are disturbed on a routine schedule; there is a plethora of handling equipment that enters and exits the tank at different times. Even though they may still adversely react to these items, their reaction could be significantly less than their wild-type counterparts. The fish then do not act in their labelled type because of the familiarity of the housing tank and the housing fish amongst them. Therefore, when any novel object comes into the tank, their reaction to it is different than if they were facing it individually. This ease of dispersion and lack of shoaling for both bold and shy fish would also explain why there was an even amount of bold and shy zebrafish in the two netting procedures.

As mentioned, these fish might have habituated to the use of netting and trapping. These fish are used in many different research studies that involve netting and sampling the fish. Even though there are behavioural differences, individually, they become irrelevant as a collective group, and the presence of boldness or shyness diminishes when they congregate together in the tank. Many passive trapping methods rely on the individual's intentional choice to investigate the trap; therefore, shyer individuals who explore less are more likely to reject the trap. Nevertheless, both bottle traps, which are Passive Sampling Methods, sampled a reasonably even distribution of the bold and shy behavioural type.

Another finding was that the bold and shy fish acted the way bold and shy animals behave in the literature (Sih et al. 2004: Sih. 1980: Riechert & Hedrick. 1993: Huntingford. 1976: Boon et al., 2008: Groothuis & Carere, 2005: Garamszegi et al. 2009: Reale *et al.* 2000: Harcourt et al. 2009: Wilson et al., 2011; Overli et al. 2005: Stuber et al. 2013). This present study then supports this readily cited finding that locomotor activity and exploratory behaviour is positively correlated with and is within the suite of behaviours related to boldness. Looking at the NTT, the One-way Analysis of Variance showed no significant difference with the sampling methods and the behavioural measures. Suggesting that there was fish sampled from passive methods did not behave differently in the NTT compared to those sampled by active methods But, there was a significant difference between the labelled bold and shy fish and the dependent measures of the NTT. Suggesting, those labeled bold exhibited different behaviours in the NTT than those labeled shy. These behaviours are consistent with the behaviours from other NTT studies where it has been established that those who are quick to enter the upper portion of the tank and spend more time there are considered bolder than those who spend more time near the bottom of the tank (Egan et al., 2009; Reider & Connaughton, 2015, Blaser & Rosemburg, 2012). This study also supports other NTT measures, such as freezing bouts, and the number of crosses. There are differences in the layout of the NTT used in this study compared to those used in other studies. The present study used a novel tank that was divided into three different sections: a bottom third, middle third, and a top third. However, in some studies, the Novel tank varies in how it is divided (Egan et al. 2009; Rosemburg et al. 2011 ; Levin, Bencan & Cerutti, 2007; Sackerman et al., 2010).

The ET was used to label the fish either bold or shy, depending on the latency to emerge. This was done because the ET is a reliable test for the assessment of individual boldness (white et al. 2013; Burns. 2008; Brown & Braithwaite. 2004). The latency relates to boldness because a bolder animal is not as anxious about a new environment and would be more likely to venture out of the safe/enclosed refuge area, which is sparse of any predation or risk, to a riskier and threat-imposing area. There can be issues with the ET in whether the desired measured variable is assessing boldness, which would be because fear and anxiety are attributed to remaining leaving the refuge area, or if it is a general lack of curiosity that causes the fish to leave. On that same notion, the understanding of correlated behaviours puts into question whether curiosity is linked with bolder animals because of the already prevalent correlations to predation, activity, foraging, and exploratory behaviour. Additionally, in some ETs, there are more measurements than just latency to emerge. For example, in a study by Miller et al. (2006) that used rats recorded multiple other measures other than latency to emerge, such as latency to approach within one body length of the door or latency to protrude the head through the door (Miller et al., 2006). These additional measures can relate even further levels of boldness or curiosity. The use of just one measure for this present study was done for the ease of the median split. Then comparisons using that could be made for the animal's boldness tendencies against other correlated behaviours.

The studies mentioned also discussed the effect of hunger levels on the performance on the ET and the effect of huger on evaluating boldness. However, the fish used in this present study were fed routinely to avoid this confounding variable.

Other areas that could be confounding variables are the size of the fish and the sex of the fish. The individual fish were not sexed or sized. This study was solely investigating the sampling bias of the specific lab sample of the home population. With that, the inclusion of the size and sex is not necessary. However, in further studies, the inclusion of size difference or sex and their effect on the behavioural traits and sampling could be investigated. However, there have been studies that have shown sex and size between zebrafish and behavior (Malmvist & Hansen. 2001; Way et al. 2015; Brown &Braithwaite. 2004)

The presence of systematic trait bias will have a significant impact on inferences made from the selected research (Biro P A. 2013). Having studies that focus on this inherent bias that behaviour puts onto sampling methods will further legitimize studies for any animal model. One of the main reasons people conduct studies is to make some inferences regarding a specific condition onto a population. For researchers to make those inferences effectively, there needs to be an aspect of randomization in their sampling, allowing for every element that is present in the population to have an equal chance to be obtained in the sample. This randomization allows the research to make better inferences and have better predictive validity. However, the present study has limitations that hinder its predictive validity. Possible confounding variables are that study looked at adult zebrafish that were laboratory-housed and sampled those fish from a home laboratory tank; the zebrafish went through the behavioural assays in succession; the size, sex, and age of the zebrafish were not analyzed; and the choice of using the specific sampling methods and not others. All these factors could pose a risk to a generalized inference on the sampling of adult zebrafish. Even though reasonably Passive Sampling Methods were used, many different other Passive Sampling Methods could be used and varied. Similarly, the housing tank could be varied. There could be further tests revolving around the dimensions of the housing and how that may affect shoaling tendencies. The behaviour of the fish could show that a housing area that resembles a wild-life environment, a bigger home space, could cause the fish to shoal differently, which could show a different result when using passive bottle traps. This change could also affect the Active Sampling Methods because the depth of the tank differentiated them. The fish could also be sampled from a novel tank instead of their home tank. A study by Wilson et al. 2011 that sampled bluefin sunfish using angling techniques and seines found that seines captured a diverse range of bold and shy and found that, counter-intuitively, angling techniques captured more timid and shyer sunfish. Subsequently, they ran another angling technique sampling test on those sunfish that were caught by the seine, but they transported them to a novel pool environment and fished from that pool. The angling technique on the seine fish in a novel environment sampled more bold fish than the initial angling sample. This suggests that in a familiar environment, shyer sunfish are not as wary of potentially risky food or objects, but the opposite is seen in a new environment. A future study could implement the same methods for laboratory-reared zebrafish and place a subsection of the home fish into a novel tank and sample from that tank. A further test can look at other situational contexts that may change the consistent behaviour seen in the fish.

This investigation sought to shed some understanding of the innate bias sampling techniques put onto the collection of bold and shy animals. In pursuit of this outcome, it demonstrated that behavioural types bold and shy do vary in laboratory housed fish. However, bold fish do not seem to be overrepresented when sampled by bottle traps. With that knowledge, the behavioural types and the methods used to sample them are important considerations for future experimental designs, as well as general housing conditions. For significant inferences to be made in zebrafish research and in any research on an animal model that is sampled from a population, these fundamental issues must be overtly addressed. The outcome of a lack of understanding could affect the validity of the study.

The zebrafish has the potential to be a highly valuable animal model, especially given its practicality, robust behavioural responses, and systems homology to humans (Gerlai, 2010). Consequently, there is a need for further optimization of zebrafish husbandry and experimental designs to ensure the outcomes and confidence in the zebrafish as an established testing animal in behavioural research.

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**Figures**

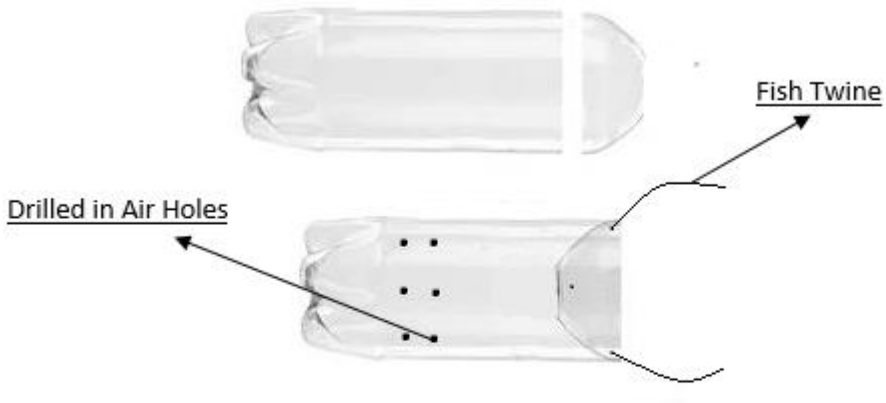


*Figure 1.* The bottle traps. A 502.8ml soda with the top removed (14cm tall and 7cm wide) and the red lego brick that was 2.0cm wide and 1.7 cm tall. These traps were classified as Passive Sampling Methods and were laid at the bottom of the tank.

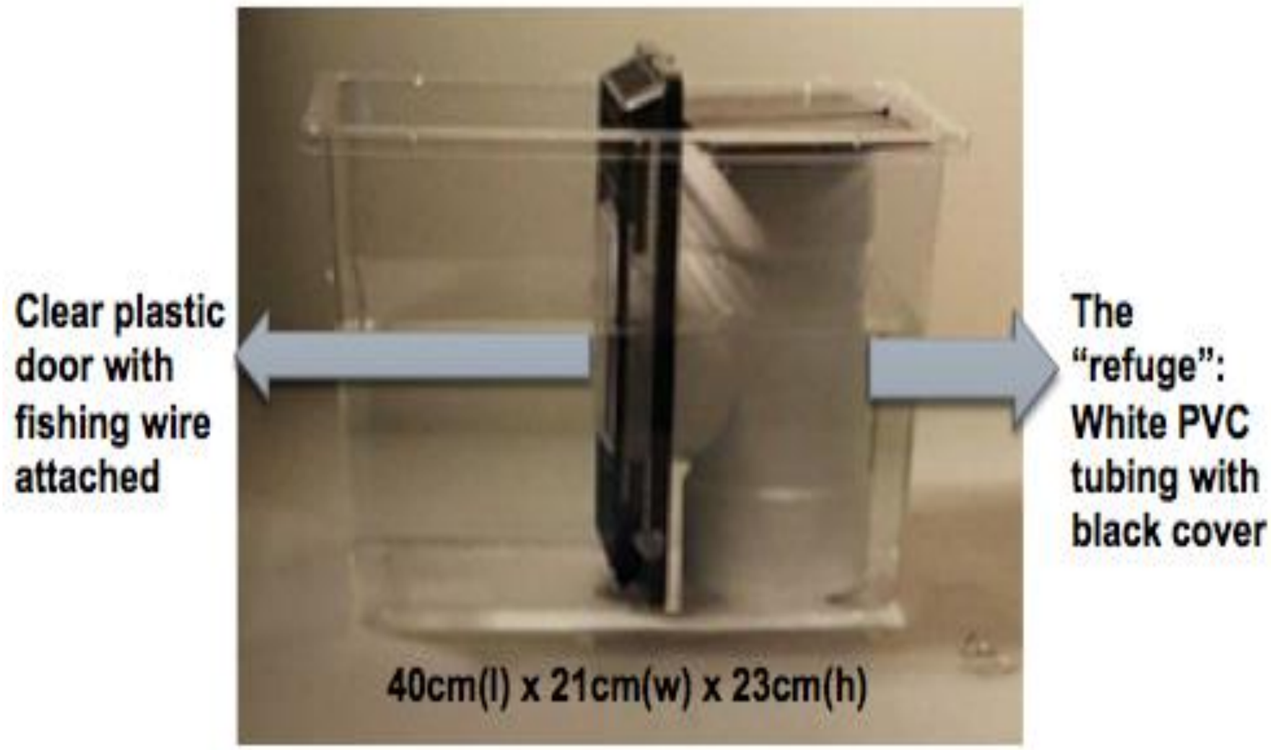


*Figure 2.* The Emergence test. Emergence test apparatus used to separate the fish into either bold or shycategories. The fish were placed inside the refuge for an acclimatization time of 2 minutes and the latency to emerge was measured as soon as the door was opened.

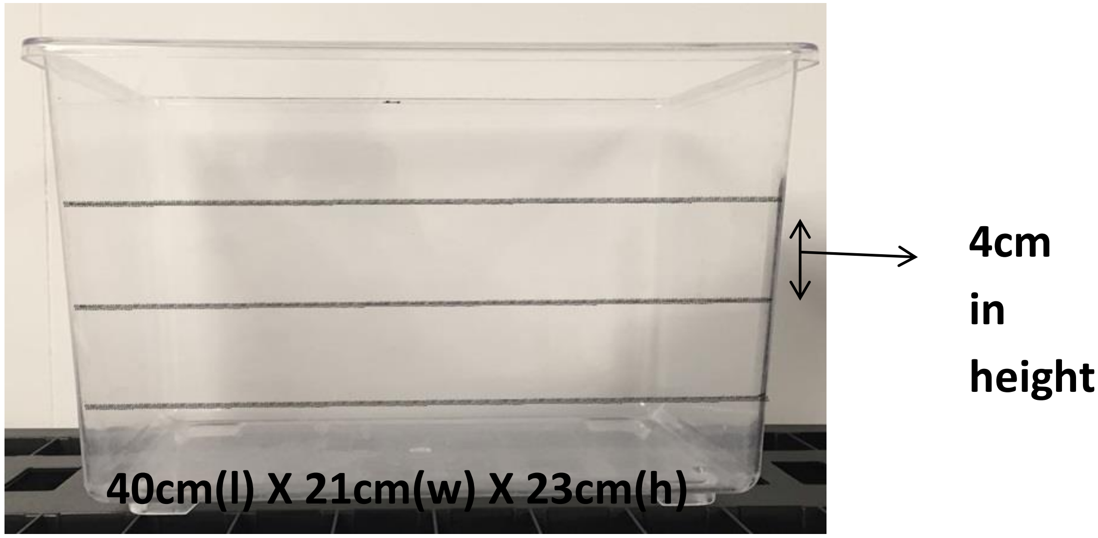


*Figure 3.* The Novel Tank Test. Novel Tank test was used to assess different measures related to the anxiety and locomotor activity

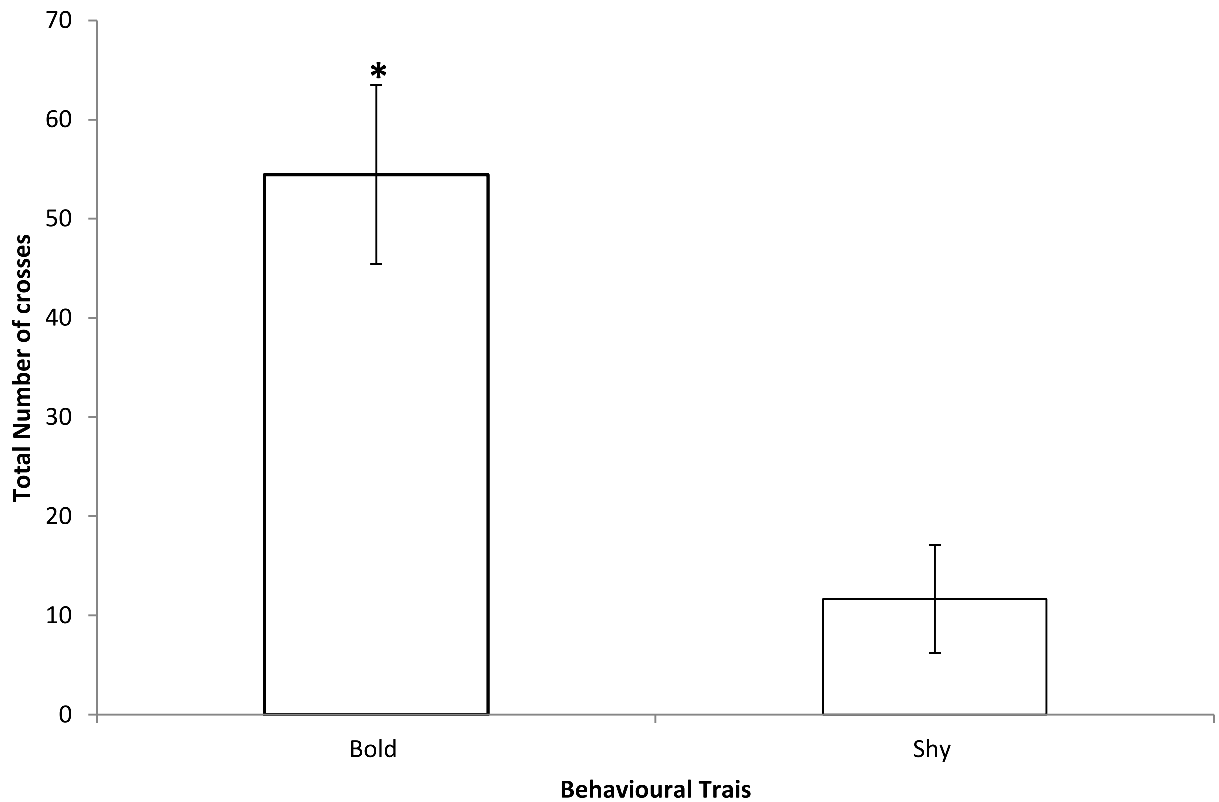


*Figure 4.* The mean (+/- S.E.M) total number of crosses for the bold and shy fish, which shows the overall activity difference between the two labelled behaviors. (* indicates a significant difference between the two groups α < .05)

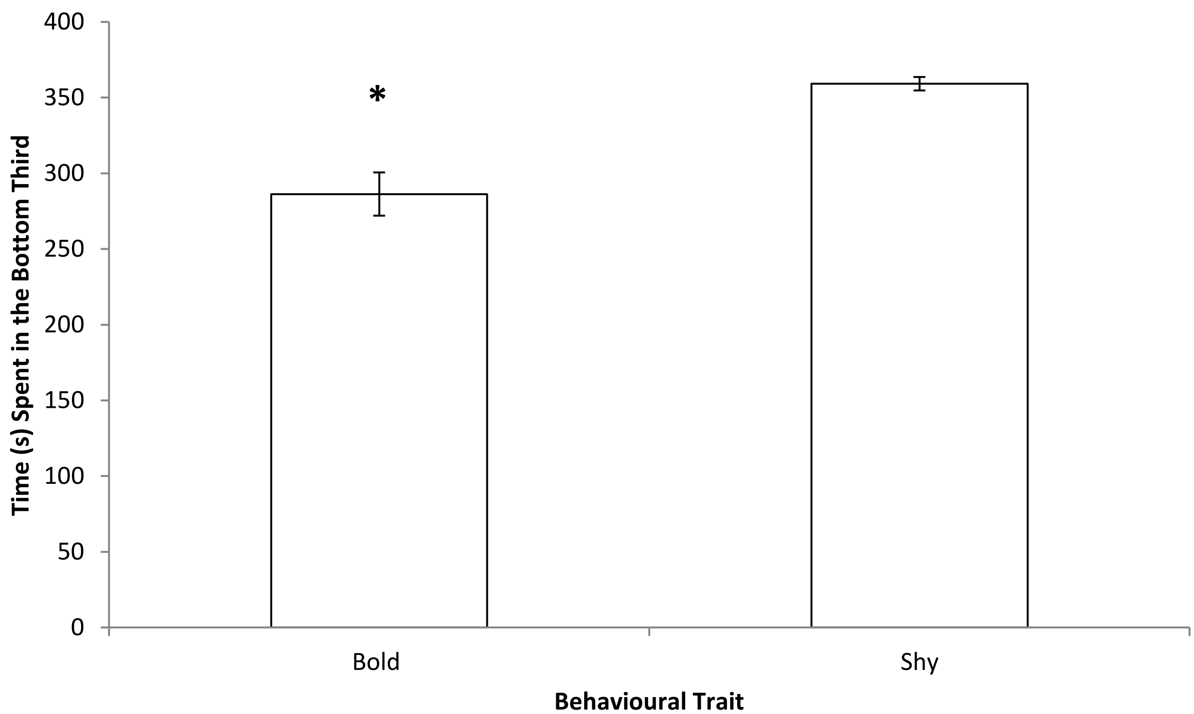


*Figure 5.* The mean(+/- S.E.M) time (s) spent in the bottom third for both the bold and shy fish, which shows the overall difference in anxiety to a novel area between the two labelled behaviours. (* indicates a significant difference between the two groups $\alpha < .05$)

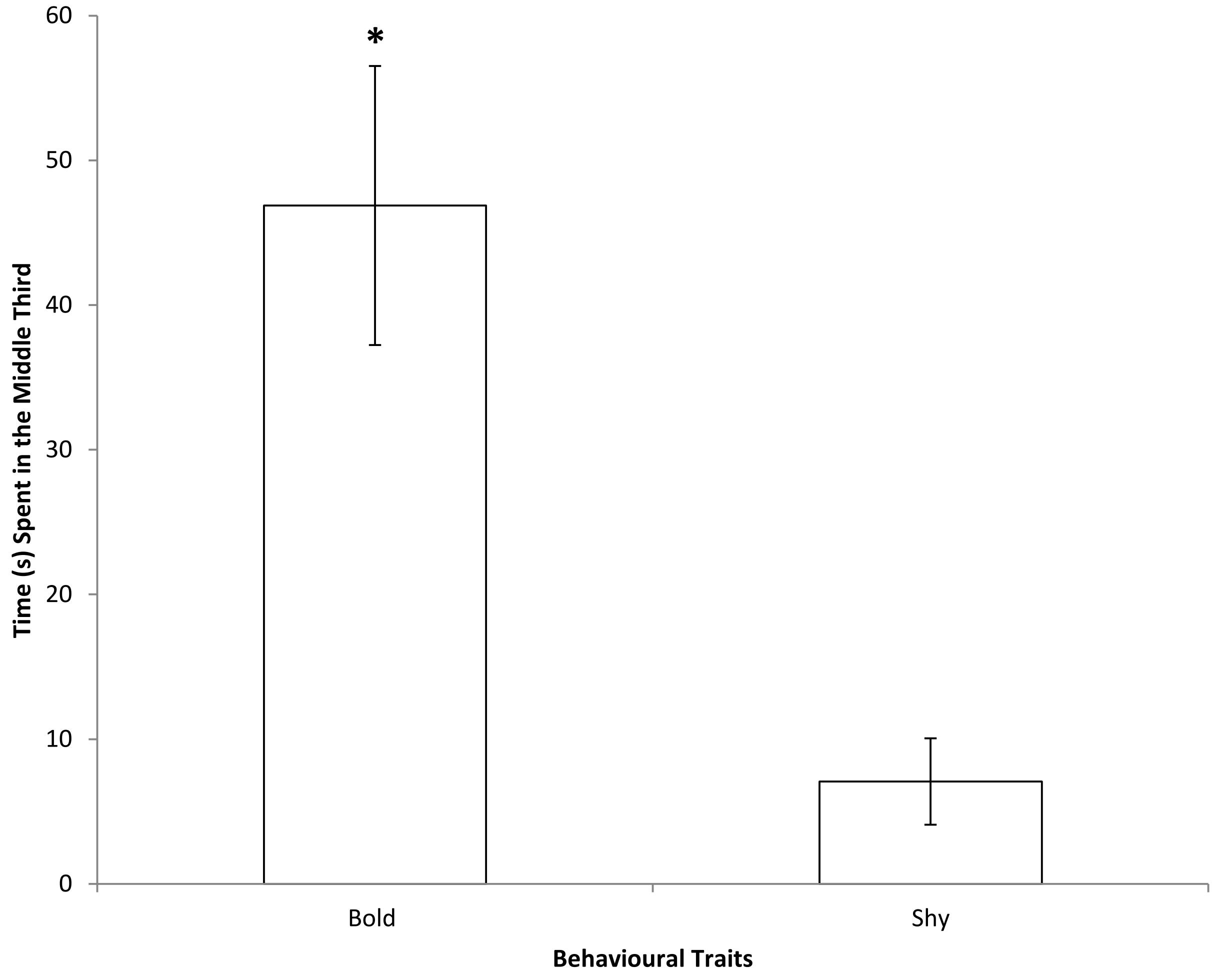


*Figure 6.* The mean (+/- S.E.M) time (s) spent in the middle third for both the bold and shy fish which shows the exploratory nature and lack of anxiety to novel areas between the two labelled behaviours. (* indicates a significant difference between the two groups α < .05)

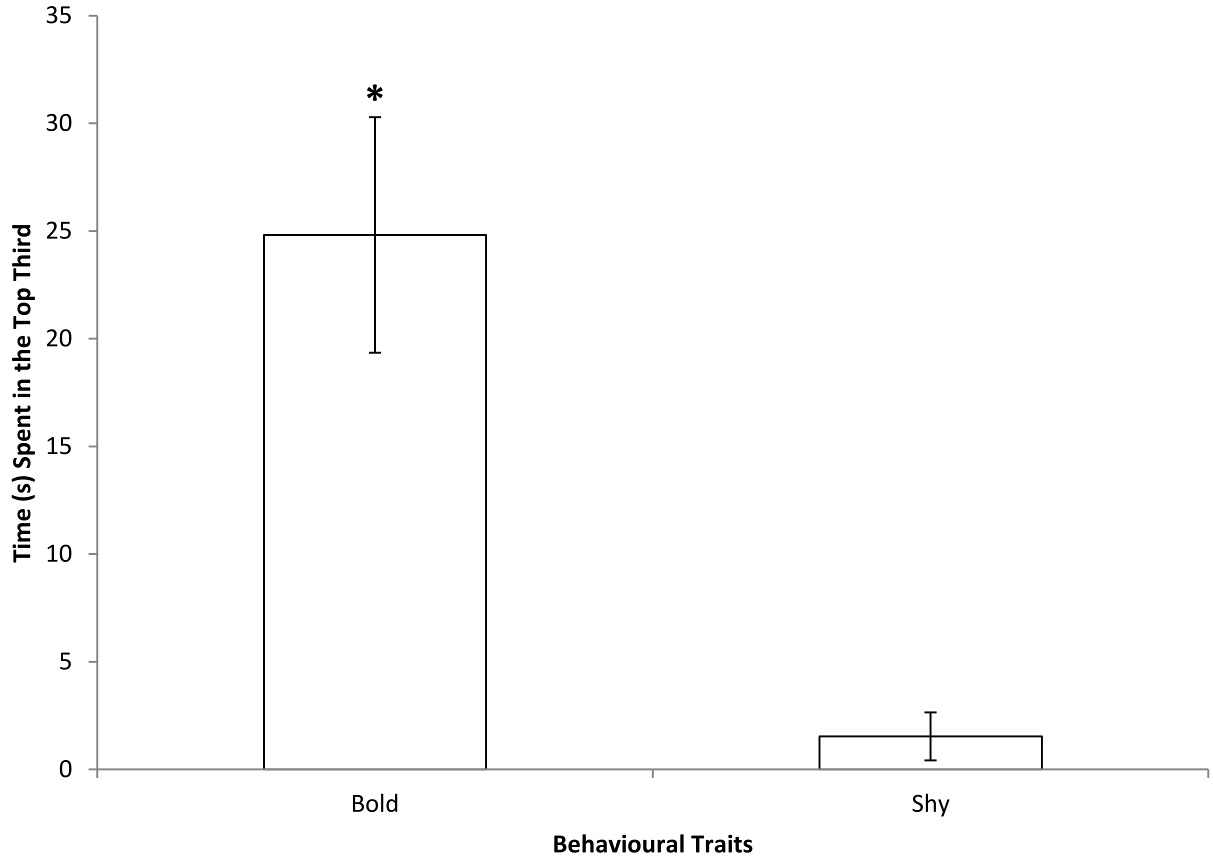


*Figure 7.* The mean (+/- S.E.M) time (s) spent in the top third for both the bold and shy fish, which shows the exploratory nature and lack of anxiety to novel areas between the two labelled behavours. (* indicates a significant difference between the two groups α < .05)

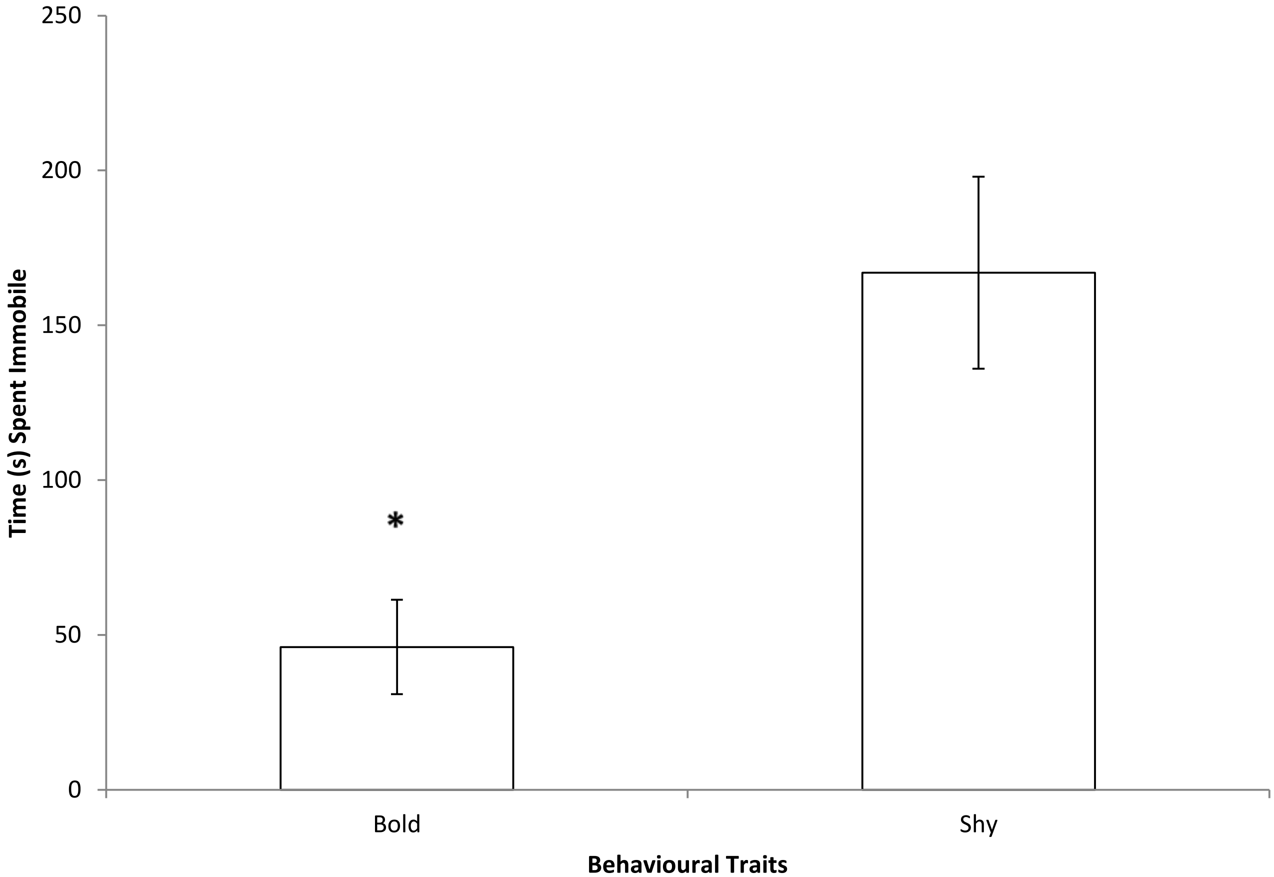


*Figure 8.* The mean (+/- S.E.M) time (s)spent immobile for both the bold and shy fish, which shows the anxiety level of the fish in respect to a novel area between the two labelled behaviors. (* indicates a significant difference between the two groups $\alpha < .05$)

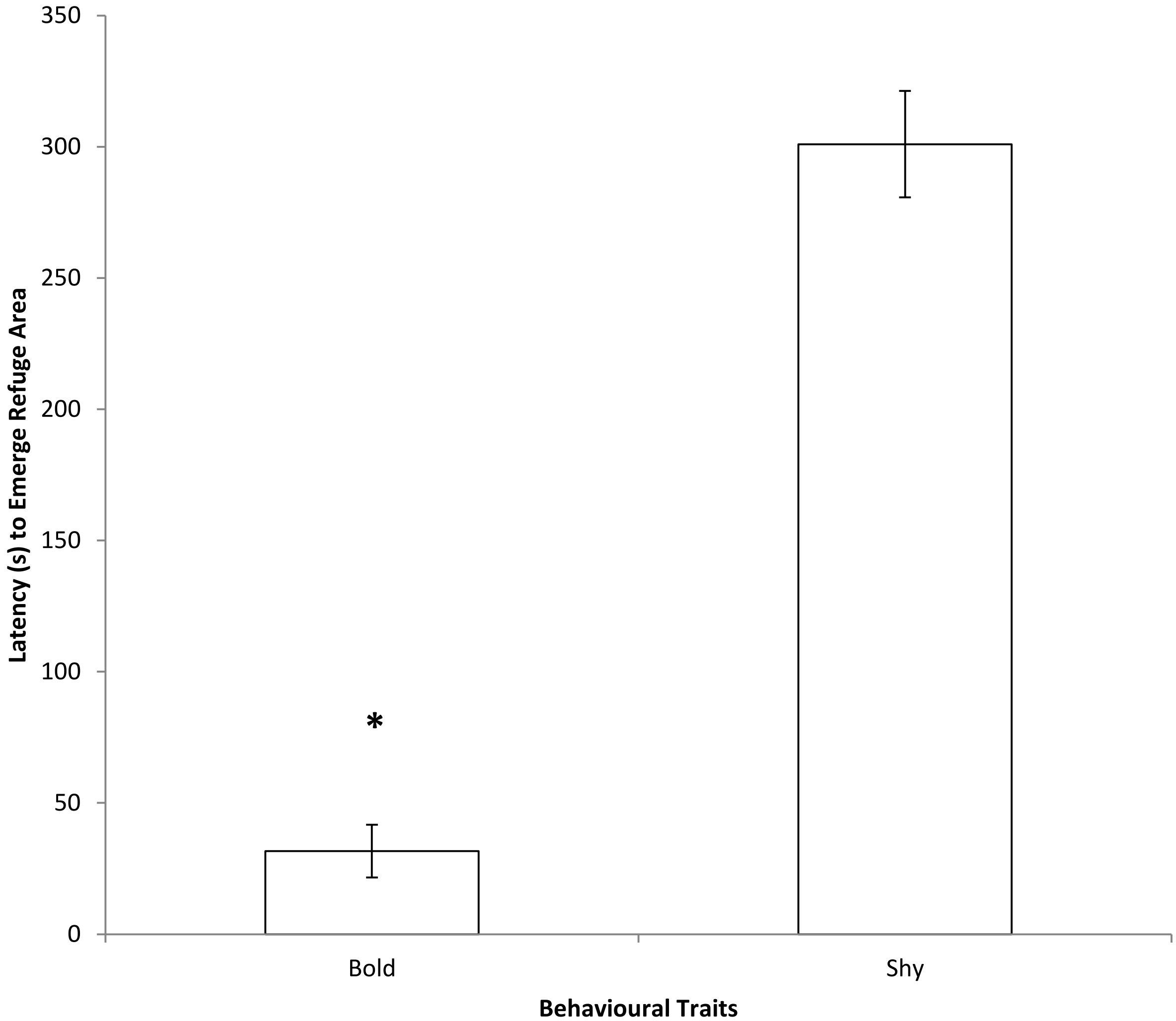


*Figure 9.* The mean(+/- S.E.M) latency time (s) to emerge out of the refuge area for both the bold and shy fish. This test was used to split the sample of fish into the two categorical variables of “bold” and shy (* indicates a significant difference between the two groups α < .05)

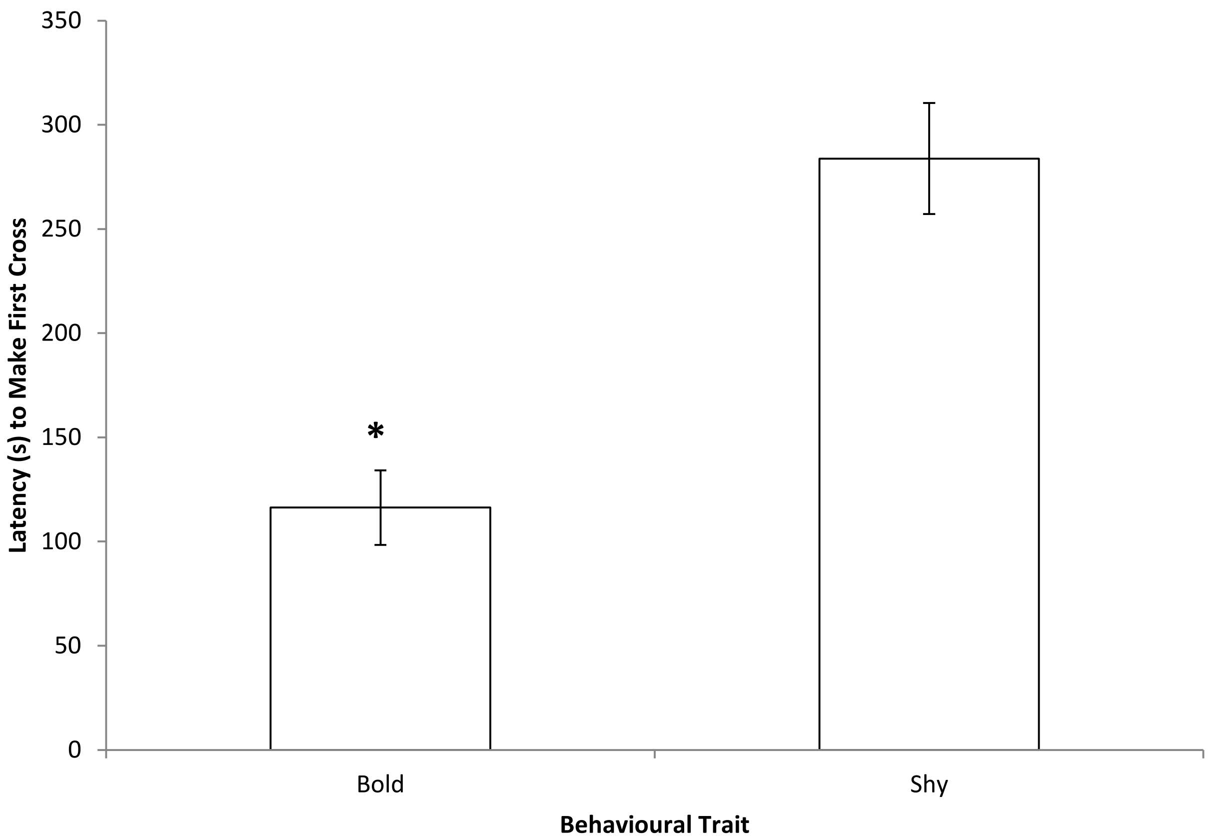


*Figure 10.* The mean (+/- S.E.M) latency time (s) to make the first cross for both the bold and shy fish, which shows the exploratory nature and lack of anxiety to novel areas between the two labelled behaviors (* indicates a significant difference between the two groups α < .05)

**Tables**

*Table 1. One-way ANOVA comparing Sampling methods for Dependent Measures*

| | F value | P value |
|---|---|---|
| Total Crosses | F (3, 36) = .672 | 0.575 |
| Time (s) Spent in The Bottom Third | F (3, 36) = .188 | 0.904 |
| Time (s) Spent in The Middle Third | F (3, 36) = .470 | 0.705 |
| Time (s) Spent in The Top Third | F (3, 36) = .520 | 0.671 |
| Time (s) Spent Immobile | F (3, 36) = .1.584 | 0.210 |
| Time (s) to Make First Cross | F (3, 36) = .893 | 0.454 |
| Latency (s) to Emerge | F (3, 36) = .548 | 0.652 |

*Significance value a<.05*

*Table 2. Table of observed and expected bold and shy fish in each of the sampling methods*

| | Active Sampling Method 1 | Active Sampling Method 2 | Passive Sampling Method 1 | Passive Sampling Method 2 | Total |
|---|---|---|---|---|---|
| bold | 5 | 4 | 5 | 6 | 20 |
| Expected bold | 5 | 5 | 5 | 5 | 20 |
| Shy | 5 | 6 | 5 | 4 | 20 |
| Expected shy | 5 | 5 | 5 | 5 | 20 |
| Total | 10 | 10 | 10 | 10 | 40 |

*Table 3. Combined overall means ± SEM for Dependent Measures in NTT and ET against sampling methods*

| Measure | Active Sampling 1 | Active Sampling 2 | Passive Sampling 1 | Passive Sampling 2 |
|---|---|---|---|---|
| Number of crosses Total | 30.5+/- 10.5 | 34.3 +/- 11.6 | 46.2+/- 17.5 | 21.2+/- 9.3 |
| Time spent in Bottom Third (s) | 326.7 +/- 15.4 | 326.8 +/- 12.8 | 310.1 +/- 25.2 | 327.3 +/- 21.9 |
| Time spent in Middle Third (s) | 18.6 +/- 7.6 | 21.2 +/- 6.8 | 34.9 +/- 12.7 | 33.3 +/- 17.8 |
| Time spent in Top Third(s) | 12.5 +/- 7.0 | 13.2 +/- 4.7 | 19.4 +/- 10.1 | 7.5 +/- 3.3 |
| Time spent immobile(s) | 60.7 +/- 36.5 | 102. 3 +/- 41.1 | 89.1 +/- 39.3 | 174.1 +/- 36.4 |
| Latency to Emerge (s) | 179.2 +/- 48.6 | 211.3 +/- 49.9 | 146.2 +/- 50.0 | 128.5 +/- 49.2 |
| Latency to make first cross (s) | 198.4 +/- 40.7 | 219.3 +/- 44.7 | 145.8 +/- 41.8 | 236.7 +/- 39.3 |

*Mean +/- S.E.M of each dependent measure as a function of sampling type*

*Table 4. Combined overall means ± SEM for Dependent Measures in NTT and ET against bold and shy traits*

| Measure | bold | Shy |
|---|---|---|
| Total Number of crosses | 54.5 +/- 9.0 | 11.7 +/- 5.5 |
| Time spent in Bottomthid (s) | 286.2 +/- 14.3 | 359.2 +/- 4.4 |
| Time Spent in Middlethrid (s) | 46.9 +/- 9.6 | 7.1 +/- 3.0 |
| Time Spent in the Topt hird (s) | 24.8 +/- 5.5 | 1.5 +/- 1.1 |
| Time Spent Immobile (s) | 46.1+/- 15.2 | 167.0 +/- 31.0 |
| Latency to Emerge (s) | 31.7 +/- 10.1 | 300.9 +/- 20.3 |
| Latency To Make First Cross (s) | 116.3 +/- 17.9 | 283.8 +/- 26.7 |

*Mean +/- S.E.M of each dependent measure as a function of Behavioural Trait*