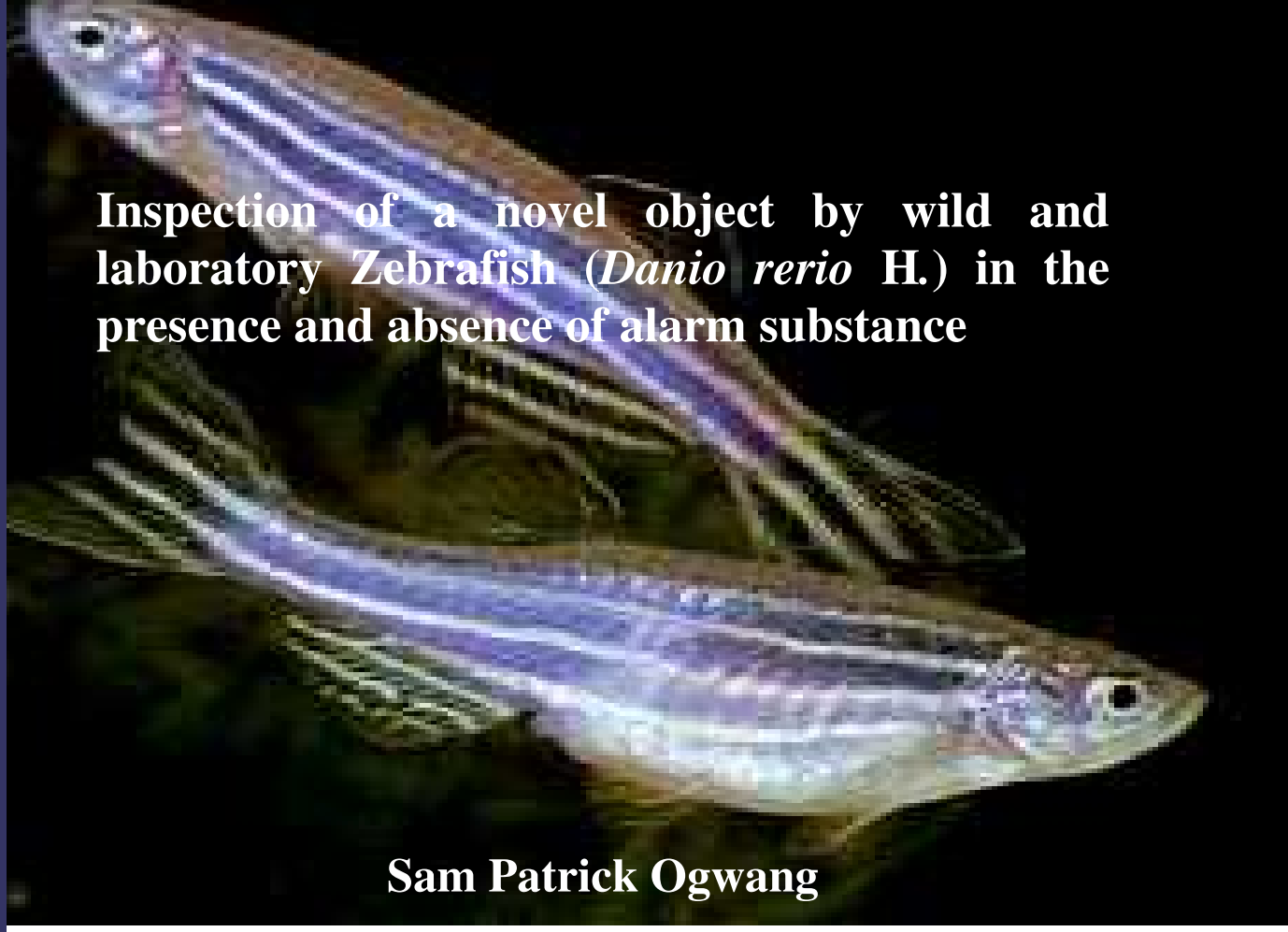

Inspection of a novel object by wild and laboratory Zebrafish (*Danio rerio* H.) in the presence and absence of alarm substance

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By

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ABSTRACT

Zebrafish (*Danio rerio*) is a small Ostariophysian cyprinid fish with no special body armour, which makes them vulnerable to predation. They possess chemical alarm pheromones (Schreckstoff) in the epidermal club cells, which, if released through damaged skin, elicit fright responses in conspecifics. Evidence suggests that domesticated fish are bolder, and thus approach potential predators less cautiously. Whether this boldness persist when alarm substance are involved is the question which this thesis was aimed to answer. The behaviour of wild and laboratory zebrafish towards a novel object was examined in the presence and absence of alarm substance with regards to four behaviour measures; novel object approach, freezing, shoaling and aggression. Fish were subjected to four different treatments; alarm substance, alarm substance plus novel object, distilled water plus novel object and distilled water (the control). Trials were recorded on video-tapes and analyzed. Results showed significant differences between the strains in latency to approach novel object, freezing and shoaling but no difference in aggression. The laboratory fish approached the novel object earlier and froze longer than the wild fish. Wild fish shoaled longer than lab strain and most often in groups of three in contrast to the lab strain where shoals of two were as frequent as shoals of three fish. The presence of alarm substance increased shoal cohesion in both strain but more in the wild strain. Alarm substance also increased freezing duration in the lab strain but not in the wild strain. No effect of novel object or alarm substance was seen in aggression and there was no difference between strains. The presence of a novel object increased shoaling in both strains but more in the wild strain. The results suggest that laboratory zebrafish are bolder than wild strain. The results further adds to the findings that alarm substance induces anti-predatory behaviour in both strains of zebrafish, indicating that the reaction to alarm substance is innate. The choice of which anti-predatory response to adopt also seems to depend on experience with predators and energetic cost of the particular response exhibited.

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1. INTRODUCTION

1.1. Background of the study

Zebrafish (*Danio rerio*) is an Ostariophysian cyprinid fish. It is a powerful model organism for the study of vertebrate neuro-anatomy and developmental biology and genetics (Moorman 2001; Kato et al. 2004; Risner et al. 2006; Wright et al. 2006a). The fish has also been widely used in ornamental public show and domestic aquariums. They adapt well in the lab, occupy relatively small tank space, and have high fecundity with rapid generation time (Korpi and Wisenden 2001; Darrow and Harris 2004). Zebrafish possess well developed classic sensory modalities; taste, smell tactile, vision, balance and hearing (Moorman 2001). The small size of *danios* coupled with their timid nature, means they are prone to predation. It is therefore argued that the social schooling behaviour of these prey fishes are a defence mechanism against predators (Dale 2001; Hamilton and Dill 2002; Wright et al. 2003; Peichel 2004; Ruhl and McRobert 2005). Among the advantages of schooling is that more eyes are available to look out for possible predator attacks. This suggests that shoaling is adaptive and signals that maintains it have probably evolved under selection (Engeszer et al. 2004).

Alarm substance (Schreckstoff)

Like other cyprinids, zebrafish possess chemical alarm substance (Schreckstoff), which is used to alert nearby conspecifics and heterospecific prey fishes about local predation risk (Hartman and Abrahams 2000; Mirza et al. 2001). Chemical signals may originate from conspecifics and heterospecific fishes or predator odour (kairomones) (Brönmark and Hansson 2000; Korpi and Wisenden 2001; Wisenden and Millard 2001; Berejikian et al. 2003), however, there is a problem of false alarm as there are several stimuli which may evoke production of this chemical in the wild (Moorman 2001). This alarm substance is released in to the environment only when the epidermal club cells are damaged through mechanical injury or predator attack (Korpi and Wisenden 2001; Berejikian et al. 2003; Brown 2003; Wisenden et al. 2004; Friesen and Chivers 2006; Speedie and Gerlai 2008). Conspecifics and heterospecific prey fishes associate this smell with the presence of a predator and thus

respond to this signal with fright reactions characterized by increased respiration, dashing, visual alertness, shelter use, shoaling, freezing, and decreased foraging, and mating activity (Brown et al. 1997; Brown and Zachar 2002; Brown and Magnavacca 2003; Huntingford 2004; Malavasi et al. 2004).

Novel potential predator and inspection

Fishes in the wild frequently encounter predators. Maximizing survival therefore requires appropriate behavioural responses to predation risk (Brown and Warburton (1999), Brown and Godin (1999), Mirza et al.(2001), Pollock and Chivers (2004) and Vilhunen (2005)). The detection of a potential novel predator is followed by inspection in a tentative salutatory approach directed towards the predator either alone or in groups (Magurran 1986; Brown and Zachar 2002; Brown and Magnavacca 2003; Malavasi et al. 2004). However, inspection is a risky behaviour (Magurran 1986; Brown and Zachar 2002) and therefore a threat sensitive trade-off between the risk of facing a potential predator and the potential benefits associated with the novel object (Brown and Magnavacca 2003; Nannini and Belk 2006), where investment in one type of anti-predator response is likely to decrease the ability to employ other types of behaviour. Fishes seem to benefit from inspection behaviour by learned recognition of predators, visual alarm signalling, acquisition of information about the predators motivation (Brown and Zachar 2002; Brown and Magnavacca 2003; Kelley and Magurran 2003) and deterring predators (Godin and Davis 1995). However, Milinski and Boltshauser (1995) argued that predators recognizes the inspector's fleeing ability and decide not to attack it, but not because of its inspection behaviour. Milinski et al. (1997) further added that inspector's traits such as size and strength would determine the attack decision made by the predator. Non-inspecting conspecifics and or heterospecifics may benefit by observing the behaviour of the inspectors and eventually also acquire information about the predator (Kelley and Magurran 2003).

Survival and anti-predator behaviour trade offs

Anti predator behaviour is costly to prey fishes in terms of energy used, and in terms of lost time for foraging and mating (Brönmark and Hansson 2000). Prey fishes are

able to trade-off anti-predator behaviours with other activities. Brown and Cowan (2000) found that finscale dace (*Phoxinus neogaeus)* deprived food for 24 or 48 hours showed no significant difference in anti-predator behaviour when exposed to chemical odour of predator fed with dace (with alarm pheromones) with those exposed to swordtail fed predator odour. Foam et al. (2005) stated that, though risky, prey fishes would continue to feed and at the same time reduce their risk to predation by increasing their vigilance towards the predator's visual cues.

Chemical versus visual cues

Effective predator detection and inspection requires a reliable source of information in space and time (Mirza et al. 2001; Brown 2003) such as chemical, visual, tactile and auditory cues (Brönmark and Hansson 2000; Dale 2001; Kelley and Magurran 2003; Lehtiniemi 2005; Mikheev et al. 2006). For glowlight tetras (*Hemigrammus erythrozonus)*, Brown and Magnavacca (2003) reported that chemical cues are the primary sources of information triggering inspection visits. They found that, the tetras took longer to first inspect a predator, and inspected in fewer numbers when exposed to chemical odour of a live convict cichlid fed tetras, regardless of light levels. Lehtiniemi (2005) found that there was a stronger and more diverse behavioural reaction of pike (*Esox lucius*) and three spine-sticklebacks (*Gasterosteus aculeatus*) in the presence of combined visual and chemical signals. Chemical signals may alert the prey of the presence of a predator, but visual cues are important in risk assessment as far as specific size, distance, shape and posture of the approaching predator is concerned (Brown and Cowan 2000; Lehtiniemi 2005). As much as chemical and visual cues convey similar information, they are not interchangeable (Brown and Magnavacca 2003). Visual cues trigger inspection but provide unreliable information as it can easily be manipulated by the predator (Brown and Godin 1999; Brown and Cowan 2000; Brown and Magnavacca 2003). Thus predation inspection by means of chemical alarm cues becomes very important at instances where visual information is impaired, especially at night or in turbid waters (Brown et al. 1997; Brown and Godin 1999; Brönmark and Hansson 2000; Brown and Cowan 2000; Kelley and Magurran 2003). Brown et al (1997) stated that chemical cues are much useful over longer distances and it has a longer period of efficiency compared to visual cues. Chemical

communication in animals have also been reported to play an important role in locating food, mate partners, kin recognition and probably in navigation among long distance migratory fishes such as salmonids (Brönmark and Hansson 2000).

Domestication

Domestication removes some selection pressure typical of natural populations and may also modify or intensify other (Price 1999; Wright et al. 2006a). Balon (1995) stated that domestication changes the life of the animal and that it is impossible to reverse domesticated organisms to their wild ancestors. Brown and Laland (2001) stated that, because of its time and energetic costs, anti-predator behaviour often degrades once the prey fish no longer experience predation pressure. Culture fishes experiences different environments from their wild counterparts such as restriction of space and migration Huntingford (2004). They receive good quality food and therefore long distance food search is unnecessary. Apart from human disturbances and unnaturally high stocking density, hatchery-reared fishes face fewer challenges, for example, reduced predation pressure (Huntingford (2004). The extent to which the behaviour of domesticated fish deviates from their wild ancestors is likely to depend on the intensity of artificial rearing (separation) (Brown and Laland 2001; Yamamoto and Reinhardt 2003; Nannini and Belk 2006). Nannini and Belk (2006) reasoned that, prey receives no benefits but keep the costs, i.e. less time to foraging and reproduction (Brönmark and Hansson 2000) by maintaining anti-predator response in the absence of a strong predatory threat. Difference in environmental experiences of wild and domesticated fish is likely to generate and shape the behavioural difference at every stage of their lives (Huntingford (2004) . Brown and Laland (2001) stated that the impoverished conditions under which hatchery fish are raised is responsible for the deficiency in all aspects of their anti-predator behaviours.

Absence of selective predation and lack of experience with predators are a consequence of artificial selection in domesticated fishes (Alvarez and Nicieza 2003; Robison and Rowland 2005) Artificial selection may changes many aspects of the life history such as growth rate, age at maturity and fecundity (Fleming et al. 2002). Price (1999) concluded that a well known behavioural modification in domesticated animals is their minimized sensitivity to changes in their environments, which is

evidenced by their response to unusual living environments and novel objects. Yamamoto and Reinhardt (2003) in their study of dominance and predator avoidance in domesticated versus wild Masu salmon (*Oncorhynchus masou masou*) showed that farmed fish activities were less affected by presence of predation risk than wild type. They attributed this to lack of predators in the hatcheries and selection for fast growth.

Robison and Rowland (2005) found that domesticated zebrafish exhibited a decline in fright response than wild strains. This suggests that domesticated zebrafish are reckless and bolder than their wild counterparts. However, naive hatchery-reared fish learn to respond to both visual and chemical predator cues after repeated experience in other species such as gobies (*Gobiusculus flavescens)*, Utne-Palm (2001) and trout (*Oncorhynchus mykiss*) (Brown and Smith 1998). Korpi and Wisenden (2001) showed that when hatchery zebrafish were conditioned to alarm cues and pike odour in a single trial they learned to fear it and reduced their activity. This single trial learning could reduce risk of predation in repeated encounters of naïve fishes with predators. Many studies support the view that domestication reduces or eliminates anti-predator behaviour in fishes. Moretz et al. (2007) stated that domestication is expected to influence boldness and aggression in fish. For example, Fernö and Järvi (1998) found that salmon (*Salmo trutta*) fry from the sea ranched environment were more risk prone than those of wild origin. Wright, et al. (2006a) also stated that anti-predator behavioural traits (inspection and shoaling) in wild and laboratory strains of zebrafish differ, and that the absence of predators means that novel objects in the laboratory pose no threat, such that inspection has no benefit and no cost if the loss of feeding time has no impact on food intake. F_2 zebrafish were found to approach a novel object more closely and stayed in its proximity more than did their wild counterparts (Wright,et al. (2006a). Malavasi, et al. (2004) demonstrated that wild juvenile European sea bass (*Dicentrarchus labrax*) tended to inspect the predator at a closer distance than did hatchery reared juveniles, and that predator induced shoal cohesion was faster in wild than hatchery juveniles. Both wild and predator naïve hatchery juvenile *D. labrax* exhibited a clear anti-predator behaviour when presented with a common eel (*Anguilla anguilla*) predator (Malavasi et al. 2004).This response was attributed to possible remnants of innate anti-predator response which might have persisted in hatchery *D. labrax* over generations (Malavasi et al. 2004).

1.2. Research problem

Studies on many species indicate that individuals can be bold or shy and that this behaviour influences much of their reaction to a number of situations (Sneddon 2003). Boldness is a behavioural trait associated with novel predator inspection and defence (Brick and Jakobsson 2002) and Brown et al. (2005). Since fishes in high predator areas are more often likely to encounter predators, than those from low-predator areas (domesticated), wild fish are expected to be more cautious and less bold than domesticated fish (Brown et al. (2005). Robison and Rowland (2005) concluded that domesticated zebrafish behaved commonly (had reduced fright response) with other fish phenotypes, and that they were less fearful (increased boldness) and orientated mostly towards the surface than their wild counterparts. Although boldness increases with domestication, do these behavioural changes and differences between wild and domesticated *Danio rerio* persist when alarm substances are involved. Further, for all its use as an experimental animal, limited literatures exists about the inspection behaviour of this species (Bass and Gerlai 2008). As fish undergoes domestication and don't experience predators, will they loose ability to react properly to alarm substances? The response of the laboratory fish to novel objects may be more uncoupled from the alarm substances, than what is expected for the wild fish. It is however also possible that the response to alarm substances is on such a basic level that it may not change during many generations.

1.3. Objective of the study

The present study aimed to answer the questions above and to examine how wild and laboratory zebrafish balances their inspection behaviour of a novel object in the presence and absence of alarm substance. Therefore, the behavioural observations resulting from this thesis work has a descriptive value in its own right regardless of the experimental outcome.

1.4. Predictions

This thesis is based on the overall prediction that the presence of alarm substance will reduce the inspection of a novel object in both wild and laboratory zebrafish. I also predicted changes in anti-predatory behaviour resulting from domestication. Previous

studies have shown that zebrafish respond to alarm substance with fright reactions (see sub title, alarm substance, page 1). Suboski et al. (1990) and Korpi and Wisenden. (2001) demonstrated that zebrafish reacts with fear to novel stimuli when presented simultaneously with alarm substance. Wright et al. (2006a) stated that anti-predator behaviour differs between lab and wild strains of zebrafish. They further found that, laboratory strains showed a reduced shoaling tendency and increased boldness compared to the wild strains. Moretz et al. (2007) also found that laboratory zebrafish were more likely to leave the vicinity of shoal mates and approached the predator more likely than the wild fish. In situations where competition for food is high and food search does not need cooperation like in the laboratory, shoaling may be maladaptive (Miller and Gerlai 2007). However, there exists variation in the anti-predator behaviour of zebrafish (Wright et al. 2003). This thesis will proceed to test the following predictions;

1. The laboratory strain will show a more bold response towards novel object than the wild strain.

2. The presence of alarm substance will increase latency to approach a novel object in both strains but more strongly in the wild strain.

3. The presence of alarm substance will increase shoal cohesion and immobility in both strains, but more strongly in the wild strain.

4. The presence of a novel object will increase shoaling and freezing in both strains.

2. MATERIALS AND METHODS

The experiment was conducted with mature wild and laboratory strains of zebrafish. Because of limited time, I started trials with wild strain while waiting for the then growing laboratory strain to reach maturity and comparable size. Fish were exposed to four different combinations of treatment with alarm substance (A), alarm substance plus novel object (A+N), distilled water plus novel object (D+N) and distilled water, the control treatment (C). No fish was reused in any of the treatments. All treatments were recorded in video-tapes and later analyzed for the differing behaviour responses.

2.1. Test fish

Wild male and female adult (three to four centimetres total length) zebrafish (*Danio rerio*) collected from a stream in Shikarpur, Coochibur-West Bengal state in India were kept in the laboratory for 13 months prior to experiment. The laboratory fish commonly called TAB but (hereafter referred to as lab strain,) had their origin from German through Hopkins laboratory before brought to University of Bergen (Norway). Fish used in this study were born on the 30th of July 2007 and fed twice daily on TetraMin flakes for four months to 2.8-3.6 cm total length before using them. Wild fish were also fed the same way. Both wild and the lab strains were transported to the experimental room and fed in a similar manner in two separate glass tanks (100 x 30x 30 cm) at the room temperature of 26^{o}C for seven days prior to the experiment.

2.2. Novel object

An orange golf ball (diameter = 4.26 cm) was used assumably as a novel object to the fish. The golf ball was chosen for its good contrast with the white test tank and because of its cosmopolitan availability to humans and absence from the natural habitat of the fish.

2.3. Experimental arena

Treatments

Each of the treatments below was planned for both wild and lab fish to be conducted with 10 replicates.

- **i)** Alarm substance only (**A**)
- **ii)** Alarm substance plus novel object (**A+N**)
- **iii)** Distilled water plus novel object (D+ N)
- **iv)** Control treatment (**C**).

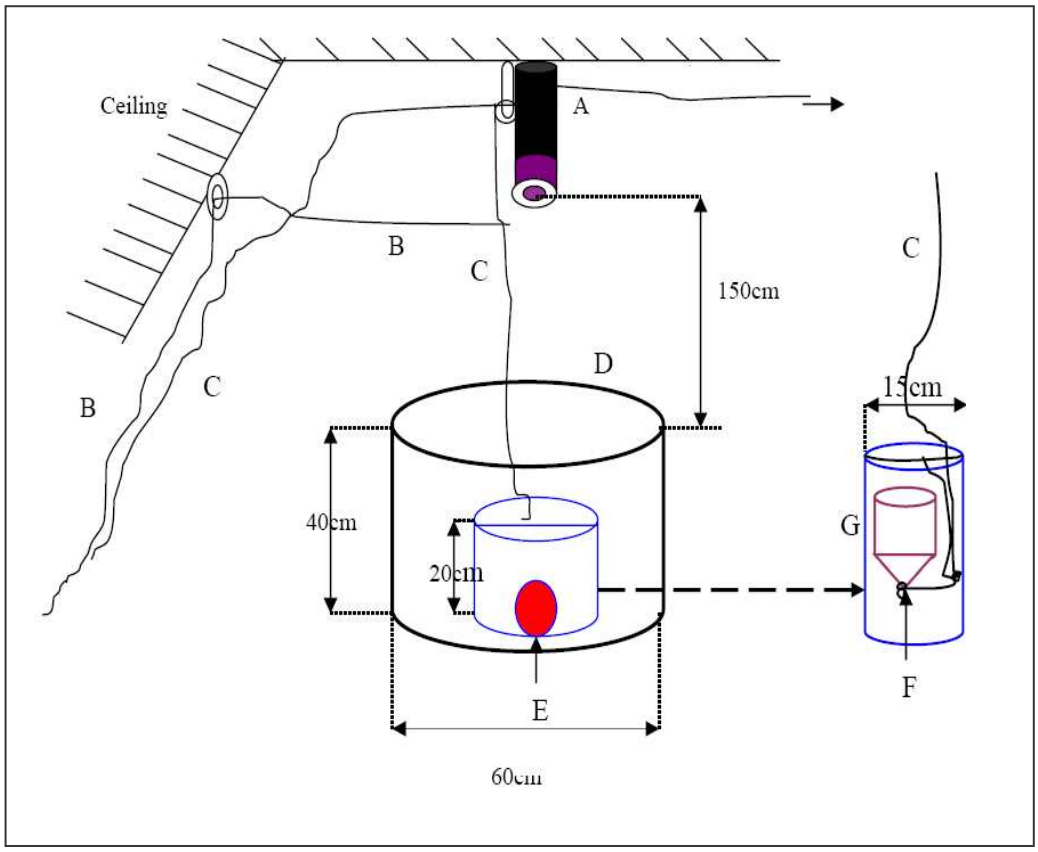

Figure 1. Showing the experimental setup. Fluorescent lamps below the test rank are not shown. (Video Camera with cable to a Monitor (A), String pulling the Cylinder off the camera view (B), Hoisting line lifting the cylinder plus Coke bottle (C), Test tank (D), Novel object (Golf ball) (E), Half cut top section of coke bottle (F), Opaque cylinder housing the bottle and hiding the ball (G).

The experimental arena (Figure 1) consisted of white cylindrical translucent plastic tanks, D (60 cm diameter, 40 cm height) filled with fresh water from the reservoir tank in the experimental room at 26^{o}C to a depth of 14 cm, equivalent of 40 litres. Because chemical substances may adhere to the tanks it was necessary to use separate tanks for the treatments with and without alarm substance. The tank used to conduct

experiment for the alarm substance and alarm substance plus novel object was marked **A** to distinguish it from the tank used for control experiments and experiments without alarm substance. An opaque white plastic cylinder, G (15 cm diameter, 20 cm height) standing at the centre of the tank hid the novel object, E and housed an inverted half cut plastic coke bottle, F used to release the alarm substance (Figure 1). Each tank was set 75 cm above three triangularly arranged fluorescent lamps which illuminated the tank from the bottom and gave better contrast of fish from the tank to aid video recordings. An automatic light regulator was installed in the room and switched light off at 2100 hour and on at 0700 hour every day to balance visual sensitivity. This was done because under normal light-darkness cycle, dark-adapted zebrafish are less sensitive to visual stimuli in the morning and most sensitive in the afternoon (Li and Dowling 1998). This is also the light rhythms under which theses fish had been kept in the laboratory prior to this study. A video camera, A (Panasonic WV-BP330/ CCTV) set vertically focusing downwards at 1.5 m from the water surface recorded the behaviour of zebrafish in the test tanks.

2.4. Preparation of Alarm substance (Schreckstoff)

The procedure followed here was modified from (Pfeiffer 1977). A donor fish were randomly picked from either the wild or domesticated strain and humanly killed clipping the fish's head in between the eyes with a pair of forceps. The weight and total length of nine wild and 13 lab fish were measured and the average of these lengths was used as reference average body length of wild and laboratory fish respectively in the quantification of behaviour. The donor fish was placed in a Petri dish and eight to ten vertical skin cuts were made on both flanks of the donor using razor blades. The fish was soaked in 200 ml of distilled water for five minutes while holding and stirring with the forceps. The extract was filtered through a 46µm plankton net secured at one end of a four centimetres diameter plastic tube to remove detached body scales. The filtrate was then diluted with distilled water to 500 ml in the case of wild strain with average weight of 0.67 g (i.e. ≈ 0.13g/100 ml) and to 250 ml in the cases of lab strain with average weight of 0.32 g (≈ 0.13g/100 ml). Waldman (1982) used a concentration of 0.65g/100 ml, but my pre-test showed a strong response to this dilution. To standardize concentrations the processed extracts were filled into panels of plastic bags each with 24 ice cubes with holding capacity of 16.7

ml and stored under refrigeration at minus 20°C until used in the experiment. Waldman (1982) found that freezing the solution did not alter the response of fish either immediately or during testing. An equal volume of distilled water used in the control experiment was processed and stored in a similar manner until used.

2.5. Experimental protocol

2.5.1. Procedures

A total of 77 experiments were conducted with schools of three fish each from the 10th of September to 10th December 2007. This school size was selected based on my own pilot experiments. Snekser et al. (2006) also used three fish in their aggregation study with normal behaviour. The original plan to conduct 80 experiments (four sets * 10 replicates each from the wild and lab strains), but the number of available fish were restricted. This resulted in nine instead of 10 replicates in the treatment sets of A+N, D+N and C from the wild strain. The decision on which experiment to run was randomized by daily picking of a tag from a box containing a series of numbered paper tags stating which treatment to do. Three fish were randomly picked from the holding tank and introduced into the test tank. The fish were allowed to acclimate for one hour before presenting the alarm substance or the novel object. Fish were not fed during this hour and during the subsequent trial of four hours.

2.5.3. Stimulus introduction

After the acclimatization the fish were introduced to the stimuli by lifting of a plastic cylinder secured to a string which ran over a pulley fixed on the ceiling directly above the tank. The remote lifting of the cylinder was done from the monitor room. This was to expose the novel object placed at the centre of the test tank to the fish without any human interference (Figure 1).

Duplicate top sections of a half cut plastic Coca-Cola bottle tightly secured with their respective black or red lids were used to introduce the alarm substance and distilled water respectively. The red lidded bottle was used for alarm substance and the black lidded for distilled water. The inverted top section containing 100 ml of water with alarm substance or distilled water for control experiment was placed floating inside

the cylinder with the lidded end below the water level (Figure 1). The lower lidded end was attached to a string joining the main string running over the pulley. This attachment was such that the cylinder and the bottle section were raised simultaneously, so that the bottle tipped and emptied its content just before the cylinder was lifted off the water surface. The red and black colours did not influence the behaviour of the fish as they could not see through the opaque cylinder covering the bottle. The choice of the bottle had no special reasons other than its availability. A third string attached midway between the pulley and the cylinder was used to pull the cylinder together with the bottle away from the view of the camera after stimulus introduction. To ensure that the alarm substance did not leak before the introduction, a blue ink solution was used to check for this before every trial. The bottle was washed thoroughly after every trial test before it could be reused in the main trial. Normal behaviour of the fish observed before stimulus release further confirmed this. The stimulus was introduced at the end of one hour acclimation period in every trial. This procedure was standardized for all sets of treatments. In the control experiments the alarm substance was replaced with an equal volume of distilled water.

2.6. Video recording and quantifying behaviour

The choice of all the parameters measured here was based on my pilot experiment conducted prior to the current study. In the monitor room located next to the experimental room there was a video recorder (*Panasonic AG 7350*) connected to the camera and a monitor (*Panasonic WV-5340*). Recordings were made for the first 10 minutes following fish introduction into the tank and from the 55^{th} min of acclimation through stimulus introduction for four hours. The initial 10 minutes recording was included to monitor possible abnormal behaviour caused by the introduction, while the last five minutes of acclimation gave the baseline behaviour prior to stimulus introduction. If fish remained motionless for over 10 minutes during the first hour of acclimation the experiment was aborted and the fish never reused. This was only seen in one trial with alarm substance from the wild strain. Eight concentric rings separated by a distance of one average body length of the fish were drawn on a transparency and fixed to the monitor screen such that the centre of the ring coincided with that of the recorded tank image. The first inner ring (one body length to novel object) was used to record an approach to the novel object. The following parameters were recorded

during the first 20 minutes of each experiment with exception of aggression which was monitored through the entire four hours until witnessed or never.

Latency to approach novel object: An approach was defined as a directed movement to within one body length (first ring) from the novel object by a single fish or by one of the fish in a group after the stimulus presentation**.** Latency was defined as the time (Seconds) until the fish first left its initial position (usually the sixth to eighth ring) after stimulus introduction to approach the novel object at the centre. Fish that moved in the opposite direction were ignored.

Frequency of approach: Frequency was defined as the number of approaches made to within one body length towards the novel object.

Shoaling duration; A shoal was defined as two or three fish coming within one body length of each other and maintaining close contact for at least three seconds. The shoaling duration was defined as the total time (Seconds) that two or three fish kept within one body length during the 20 minutes of observation. The duration was recorded separately for the two and three fish shoals. A stop clock was started in the third second of shoaling and stopped when the shoal broke up. For the two fish shoal the clock was also stopped if the third fish joined as this would form a three fish shoal group. The duration of the latter was counted as the three fish shoaling duration. The clock was started in the third second to exclude the time of shoal formation from shoaling duration.

Freezing duration: freezing was defined as when at least one fish remained motionless for a minimum of 10 seconds. Also, the time the fish went into freezing until it resumed swimming was recorded.

Latency to initiate aggression; Time from start of the trial until an attack was made on another fish followed by a chase by the attacker for over five seconds.

Frequency of aggression; The number of attacks with chasing witnessed. The frequency of aggression was monitored from initiation of attack for 20 minutes within the four hours of the experiment.

2.7. Statistical analysis

The effects of alarm substance on inspection behaviour of a novel object by wild and lab fish were statistically tested as described in the respective sub sections below. Statistical tests were done using R statistics software version **R 2.6.1** and STATISTICA version six**.** For technical reasons, all graphs (except Survival curves) were plotted using STATISTICA. Analyses generally had two factors. The two factors were "strain" with two levels (**Wild** for wild fish and **Lab** for laboratory fish) and "Treatment" with four levels (A, A+N, D+N and C (See section 2.3)).

Latency to approach novel object, and to show aggression

Latency data were analyzed using the *Survival analysis* package in R. This package analyzes the "time to event" data like time to death or time to recovery from a sickness. Not all subjects performed the event during the period of observation in this study. In other words not all fish approached the novel object or showed aggression within the 20 minutes of observation. In such cases the experiments were said to be "*Censored*" as fish were expected to approach or show aggression at a later time. This is reflected on the survival curve by a **+** at the end of each curve (see results section). For experiments that showed the expected event the censoring indicator ***status*** was coded as **one,** while it was coded as **0** in experiment where the expected event was not observed.

Frequency of approach to novel object and frequency of aggression

I used the Generalized Linear Model (GLM) of R because the data recorded did not conform to normality (Crawley 2005). In all cases, the quasi Poisson distribution was assumed.

Shoaling duration and freezing duration

Shoaling duration was tested by two separate analyses were performed. First, a Mann-Whitney U-test to reveal whether zebrafish has a tendency to shoal in small or larger groups. Secondly, a one-way and two-way ANOVA was used to test the total time shoaling (assuming a normal residual distribution) between the strains and

experimental treatments respectively. This analysis would detect any difference in general tendency of shoaling in between the experiments. Two-way ANOVA was also used to test for the differences between strains in freezing duration. For all cases of significant effects, post hoc multiple comparisons Tukey honest significant difference test (*Tukey HSD*) were performed.

3. RESULTS

Immediately upon stimulus exposure fish exploded in different directions with erratic swimming before rejoining as two or three fish in a shoal. Early in the trials when two fish were shoaling, one also see freezing at the bottom of the tank. Fish in some trials especially those exposed to alarm substance would go straight to freezing before they formed or joined a shoal. This shoal would frequently break and rejoin especially when an approach was made. The appearance of a novel object approach varied. For single fish approach, fish would leave a shoal and swim slowly either straight to the novel object or approach it in a curve and eventually go round the object while looking at it before it was joined by other fish or returned to the shoal. Aggression occurred from the middle of the first hour to the fourth hour in all trials. It begun by position displacement or interchange between the members of the shoal. Initially one member of a group would attack the other two in turns before one individual left the shoal. The remaining two fish could then engaged in a cyclic tail chase followed by biting and eventually one fish fled and the other chased it before re-directing an attack towards the individual that fled earlier. In most cases fish did not confront the dominant member. In some treatments aggression did not last for long before the group started swimming together as before, while in others, the novel object seemed to provide shelter to some one individual that chased away the other fish from the object. This indicates a form of territorial defence.

I analyzed the four behaviour responses, approach of novel object, freezing, shoaling and aggression and compared statistically and graphically the different behaviours in wild and laboratory strains of zebrafish. The fish were exposed to four different combinations of treatment with alarm substance (A), alarm substance plus novel object (A+N), distilled water plus novel object (D+N) and distilled water, the control treatment (C). To test for differences between strains I used pooled data from all trials, while for difference in treatments within a strain, individual trial data were used. Where normality assumptions were not met, data were natural log transformed.

3.1. Latency to approach novel object

The latency to approach the novel object was recorded based on when a fish in a trial for the first time moved to within one body length of the fish to the novel object. Regardless if an approach was made in groups or by a single fish this time was taken to represent the whole trial and no further approach was considered except for purpose of recording frequency of approach. Here, only data from trials which contained novel object (i.e., A+N and D+N) were considered because the approach was directed to the novel object. The laboratory strain approached the novel object earlier than the wild strain (Survival, $p < 0.05$, Table I and Figure 2). The interaction between strain and treatment had no effect on the latency to approach the novel object (Survival, $p > 0.05$, Table I).

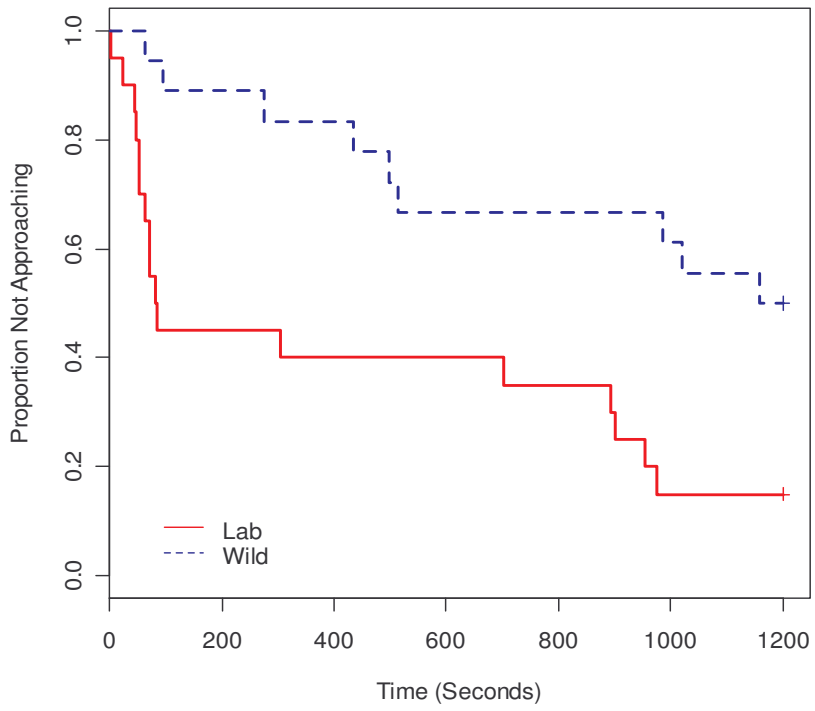

Figure 2. Survival curves showing time to initiate an approach towards a novel object within the first 20 minutes of observation. Every drop in the curve indicates a complete event, i.e., an approach. The + at the ends of the respective curves for both wild and laboratory strain indicate censoring for experiments in which no approach was made. Figure represents pooled data for all trials from each strain. The blue curve indicates wild fish and red indicates laboratory fish.

Interestingly, in both strains trials with distilled water plus novel object showed a tendency of late approach to novel object (Figure 3). The laboratory strains exposed to

alarm substance plus novel object (A+N) and those exposed to distilled water plus novel object (D+N) approached significantly earlier ($p = 0.047$ and $p = 0.029$, respectively, Table I, Figure 3) than the corresponding treatment in the wild strain. There were no significant differences between treatments within the wild or lab strains (Table I and Figure 3).

Table I. Survival analyses showing the effect of alarm substance and novel object on the latency to approach novel object by wild and laboratory zebrafish within the first 20 minutes of observation

	Source of variation	**Df**	**N**	**Z**	**SE**	**p**
General data	Wild Vs Lab strain	1	38	2.940	0.412	**0.003**
	Strain X Treatment	1	34			0.824
Wild strain	A+N Vs D+N	1	18	0.568	0.671	0.570
Lab strain	A+N Vs D+N	1	20	0.405	0.486	0.685
Wild Vs Lab	A+N	1	19	1.990	0.570	**0.047**
	D+N	1	19	2.190	0.601	**0.029**

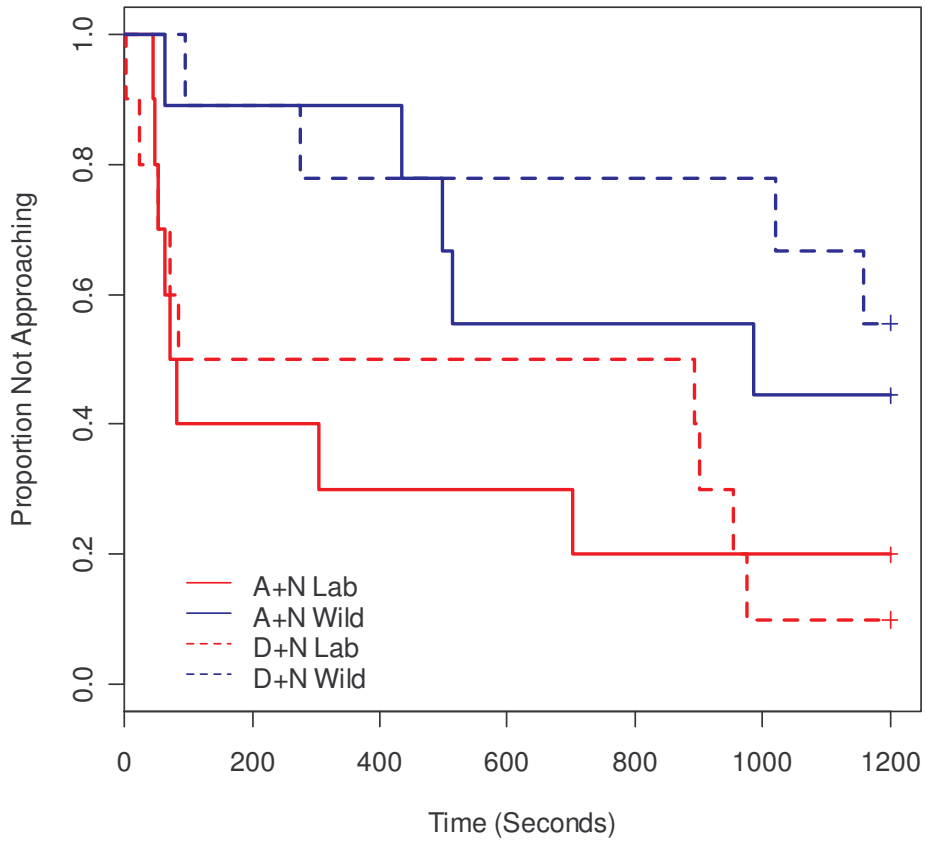

Figure 3. Survival curves showing time to initiate an approach towards a novel object by wild and lab strains with different treatments within the first 20 minutes of observation. Every drop in the curve indicates a complete event, i.e. an approach. The blue curve indicates wild fish and red indicates laboratory fish. Solid lines represent treatments with alarm substance and dashed lines are treatments without alarm substance.

3.2. Frequency of approach

Frequency was recorded as the number of approaches made to within one body length towards the novel object. If several fish approached at once, that was counted as a single event just like when a single fish approaching. The average number of approaches from all fish in a trial represented a single point per trial. The frequency of approach of the wild and laboratory strains was not significantly if different (Table II, GLM and $p > 0.05$, Figure 4). Similarly the interaction between treatment and strain had no significant effect (Table II, GLM, $p > 0.05$).

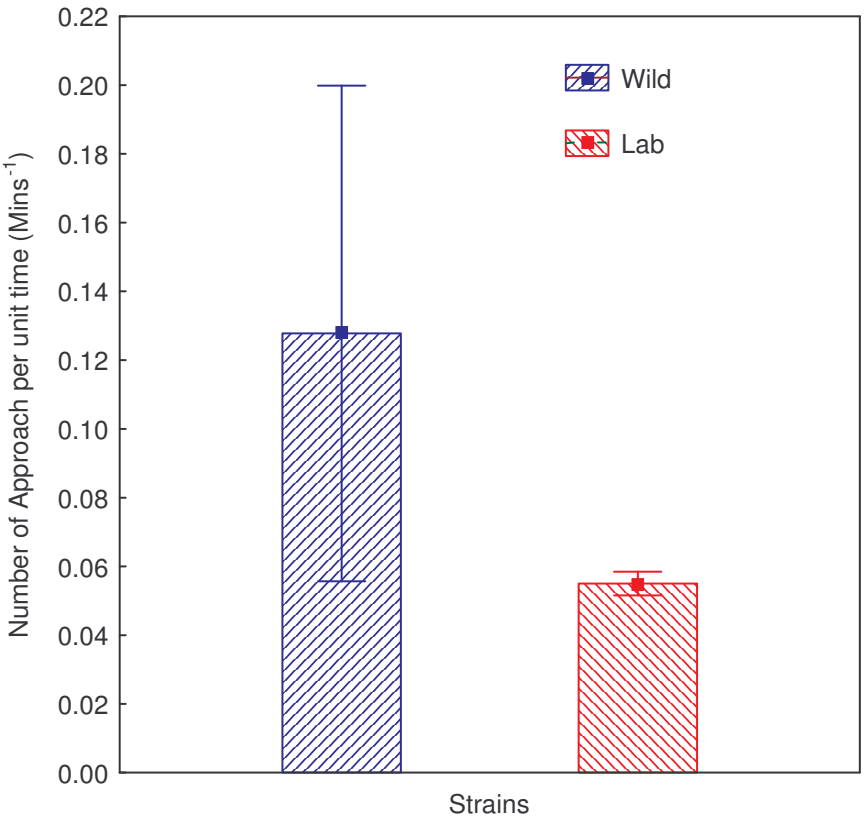

Figure 4. Mean ± SE Frequency of approaches made towards novel object within the first 20 minutes by wild and laboratory fish. Data were pooled from all treatment combinations for each strain. The blue bar represent wild strain while red is for laboratory strain.

Wild fish exposed to alarm substance seemed to approach the novel object less frequent than wild fish exposed to trials without alarm substance, (Appendix Fig A 1 and Table II), but this was not significant due to large variation in the wild strain

(Table II, $p > 0.05$). In the lab strain, the difference between treatments was small (Appendix Fig A 1 and Table II, $p > 0.05$).

Table II. Generalized linear model comparing the effects of alarm substance and novel object on frequency of approach to novel object by wild and laboratory zebrafish within the first 20 minutes of observation.

	Source of variation	Df	Residual Df	t	SE	p
General data	Wild Vs Lab strain	1	36	1.232	0.684	0.226
	Strain X Treatment	1	34	1.207	1.172	0.236
Wild strain	A+N Vs D+N	1	16	1.267	1.116	0.223
Lab strain	A+N Vs D+N	1	18		0.129	1.000

3.3. Freezing duration

The laboratory strain spent longer time freezing than did the wild strain (Table III, ANOVA $p < 0.05$ and Figure 5). There was no significant interaction between strain and treatment for freezing duration (Table III, $p > 0.05$).

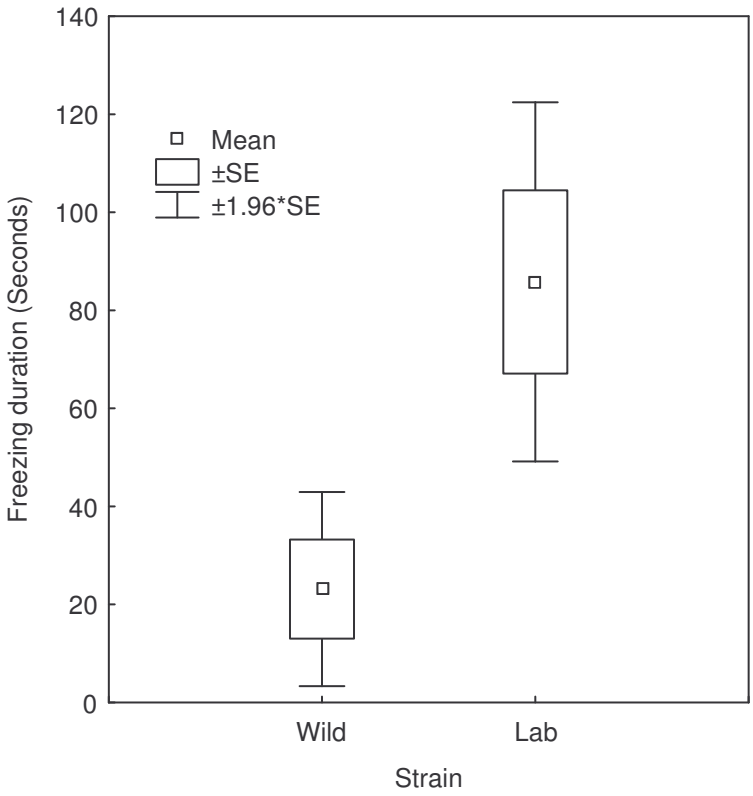

Figure 5. Mean ± SE Freezing duration of wild and laboratory fish within the first 20 minutes of observation. Figure based on pooled data from all the treatment combinations.

Table III. Two-way ANOVA comparing the effects of alarm substance and novel object on freezing duration of wild and laboratory zebrafish within the first 20 minutes of observation.

Source of Variation	Df	MS	F	p
Strain	1	8052	10.192	**0.004**
Treatment	3	3123	3.954	**0.011**
Strain X Treatment	3	1403	1.776	0.160
Residuals	68	7900		

In the wild strain, there was no difference in freezing duration between treatments (Table IV, $p > 0.05$). In contrast, there were treatment differences within the lab strain (Table IV, $p < 0.05$). In the lab strain fish exposed to alarm substance (A) froze longer than those without alarm substance (C and D+N), (Figure 6). There were no significant differences in freezing duration between any other treatments (Table IV, ANOVA, and $p > 0.05$).

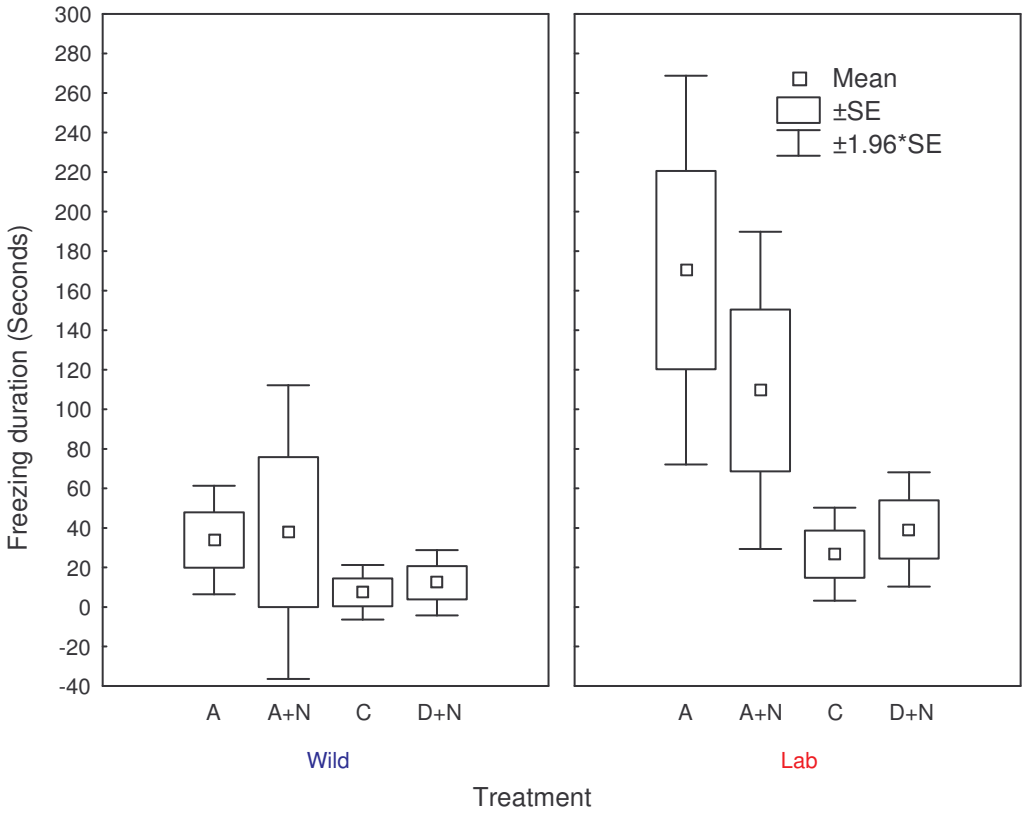

Figure 6. Mean ± SE Freezing duration of wild and lab fish under different treatments within the first 20 minutes of observation. A, A+N, D+N, and C has the same meaning as explained in the methods.

Table IV. Tukey HSD multiple comparisons of means at 95% family-wise confidence level comparing the effects of different treatments on freezing duration of wild and laboratory zebrafish within the first 20 minutes of observation.

Strains	Treatments	Difference	Lower	Upper	p
Wild	A Vs C	-26.356	-104.263	51.552	0.797
	A Vs A+N	4.0889	-73.819	81.997	0.999
	A Vs D+N	-21.578	-99.486	56.330	0.876
	C Vs A+N	-30.444	-110.376	49.487	0.733
	C Vs D+N	4.778	-75.153	84.709	0.998
	A+N Vs D+N	-25.667	-105.598	54.265	0.820
Lab	**A Vs C**	**-143.633**	**-277.348**	**-9.919**	**0.0313**
	A Vs A+N	-60.700	-190.848	69.448	0.595
	A Vs D+N	**-131.200**	**-261.348**	**-1.052**	**0.048**
	C Vs A+N	-82.933	-216.648	50.781	0.353
	C Vs D+N	12.433	-121.281	146.148	0.994
	A+N Vs D+N	-70.500	-200.648	59.648	0.471

3.4. Shoaling duration.

Shoaling was defined as two or three fish staying within one body length of another. The total time shoaling is the sum of two and three fish shoaling duration. To improve data for normality assumptions, all shoaling analyses were done with data transformed to their natural logarithms. Graphs were plotted with non transformed data for ease of visual inspection.

3.4.1. Total shoal duration

Here the total time spent shoaling as two and three fish were pooled. The two strains differed in total time of shoaling, (Table V, ANOVA, and $p < 0.05$) with the wild fish shoaling for a longer time (Figure 7). There was no significant interaction effect between strain and treatment on the time spent shoaling (Figure 8 and Table V, ANOVA and $p > 0.05$). The treatment effect was significant (Table V, $p < 0.05$). Fish in the control shoaled significantly less than fish in the rest of other treatments in both wild and lab strain (ANOVA, $p < 0.05$, Table VI and Figure 8). The other three treatments were not significantly different from each other (ANOVA, $p > 0.05$ Table VI and Figure 8).

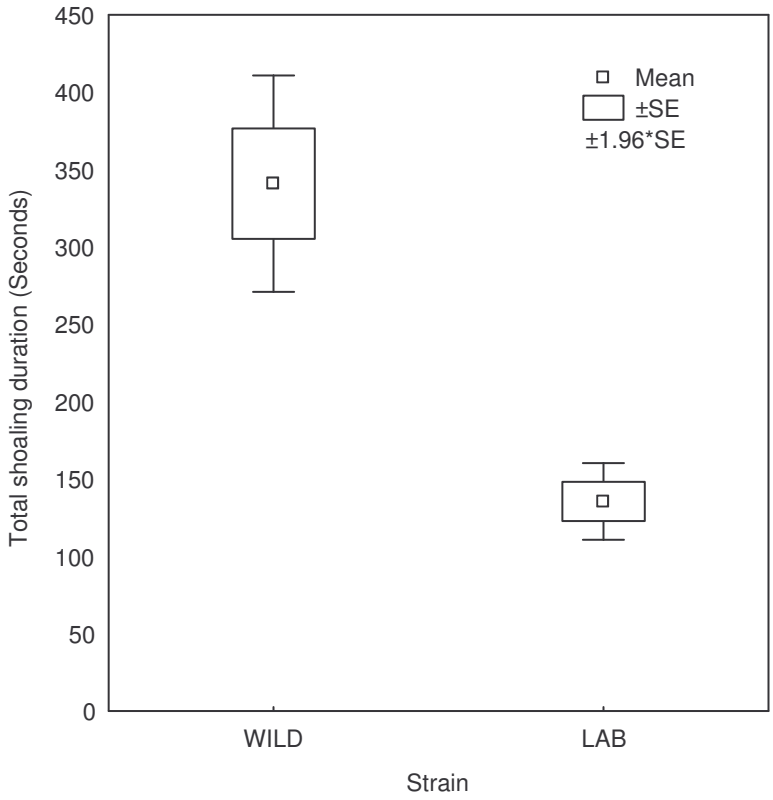

Figure 7. Mean ±SE Total shoaling duration of wild and laboratory strains exposed to different treatments within the first 20 minutes of observation. Figure based on pooled data from all the treatment combination.

Table V. Two-way ANOVAs comparing the effects of alarm substance and novel object on shoaling duration of wild and laboratory zebrafish within the first 20 minutes of observation.

Sources of Variations	**Total Shoal Duration**			
	Df	MS	F	p
Strain	1	11.941	43.435	**0.000**
Treatment	3	7.951	28.920	**0.000**
Strain X treatment	3	0.251	0.913	0.439
Residuals	67	0.275		
	Three Fish Shoal Duration			
Strain	1	16.945	31.659	**0.000**
Treatment	3	6.396	11.951	**0.000**
Strain X treatment	3	0.251	0.481	0.697
Residuals	65	0.535		
	Two Fish Shoal Duration			
Strain	1	2.969	3.406	**0.022**
Treatment	3	11.662	27.544	**0.000**
Strain X treatment	3	0.016	0.037	0.990
Residuals	65	0.423		

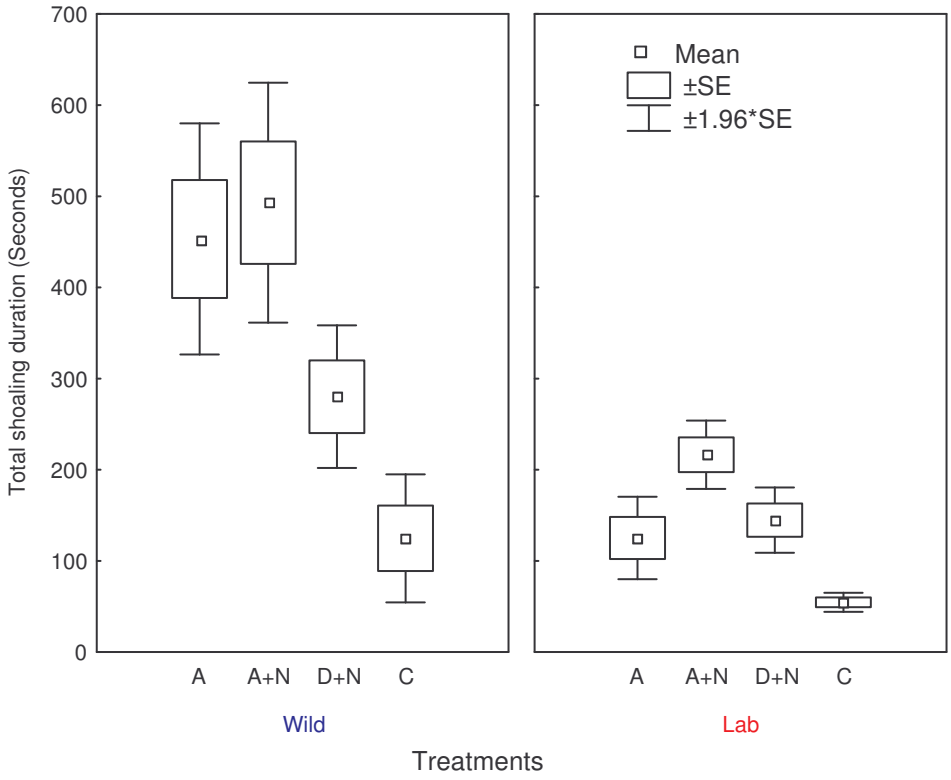

Figure 8. Mean ± SE Total shoaling duration of wild and laboratory fish subjected to different experimental treatments within the first 20 minutes of observation. A, A+N, D+N, and C has the same meaning as explained in the methods.

Table VI. ANOVA, Tukey HSD multiple comparisons of means (95% family-wise confidence level) comparing the effects of alarm substance and novel object on total shoaling duration of wild and laboratory strains of zebrafish under different treatments within the first 20 minutes of observation.

Treatments	Difference	Lower	Upper	p adjusted
Wild Strain				
A Vs A+N	0.081	-0.636	0.799	0.989
A Vs C	**-1.463**	**-2.181**	**-0.745**	**0.000**
A Vs D+N	-0.468	-1.186	0.250	0.309
C Vs A+N	**-1.544**	**-2.281**	**-0.808**	**0.000**
A+N Vs D+N	-0.549	-1.286	0.188	0.203
C Vs D+N	**0.995**	**0.259**	**1.732**	**0.005**
Lab Strain				
A Vs A+N	0.464	-0.134	1.062	0.175
A Vs C	**-0.921**	**-1.519**	**-0.323**	**0.001**
A Vs D+N	-0.039	-0.638	0.558	0.998
C Vs A+N	**-1.385**	**-1.949**	**-0.821**	**0.000**
A+N Vs D+N	- 0.504	-1.068	0.059	0.094
C Vs D+N	**0.881**	**0.317**	**1.445**	**0.001**

3.4.2. Two fish shoaling duration

In the two fish shoals, the wild strain shoaled significantly longer than the lab (Table V, $p < 0.05$, Appendix Fig A 2). Both treatment and strain had significant effects on the shoaling duration, but the interaction between the two was not significant ($p > 0.05$, Table V). In both strain, post hocks multiple comparisons test with Tukey HSD revealed a significant difference between fish exposed to the control and those exposed to alarm substance, alarm substance plus novel object and distilled water plus novel object (ANOVA, $p < 0.05$, Appendix Table A 2 and Appendix Fig A 3).

3.4.3. Three fish shoaling duration

Wild fish shoaled significantly longer than the lab strain ($p < 0.05$, Appendix Table A 2 and Table V). In the wild strain, fish exposed to alarm substance and those exposed to alarm substance plus novel object shoaled significantly longer than those in the control while in the lab strain only fish in treatment with alarm substance plus novel object shoaled significantly longer than the control group (Appendix Table A 2 and Appendix Fig A 5).

3.4.3. Two fish versus three fish shoaling duration

Wild fish shoaled significantly (Mann-Whitney U test, $p < 0.05$) more as three than as two fish (Table VII and Figure 9) and all trials significantly induced cohesion as two fish shoal but only trials with alarm substance (A and A+N) increased cohesion as three fish shoal (Appendix Table A 3 and Appendix Fig A 6). In the lab strain, there was no significant difference in shoaling duration between groups of two or three fish (Table VII and Figure 10). Lab fish exposed to alarm substance plus novel object shoaled significantly in groups of two longer than the fish in the control. However, when shoaling as three fish, no treatment had significant effect (Appendix Table A 4 and Appendix Fig A 7).

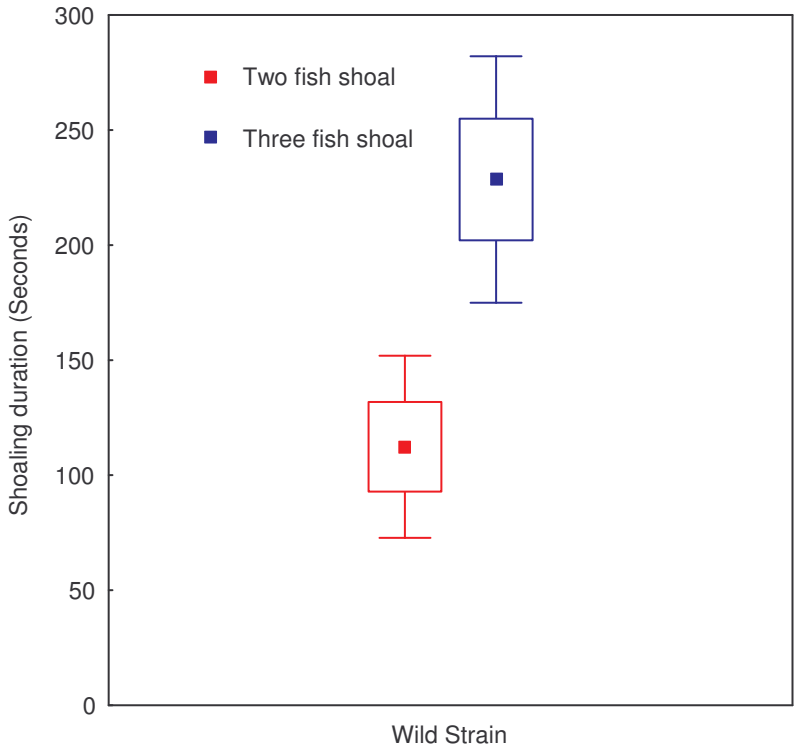

Figure 9. Mean ± SE Shoaling duration of wild fish in groups of two and three fish within the first 20 minutes of observation. The blue box represent fish shoaling in groups of three and red is for fish shoaling in groups of two.

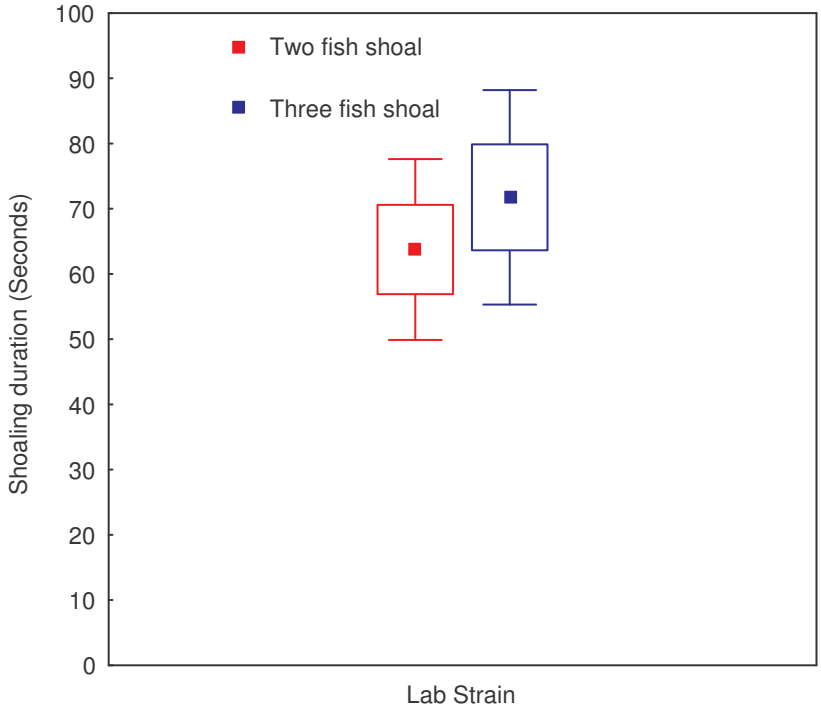

Figure 10. Mean ± SE Shoaling duration of lab fish in groups of two and three fish within the first 20 minutes of observation. The blue box represent fish shoaling in groups of three while red represents fish shoaling in groups of two.

Table VII. Mann-Whitney U Test. Shoaling duration in group of two and three fish by wild and laboratory zebrafish within the first 20 minutes of observation. Marked tests are significant at $p < 0.05$

	Rank Sum (Two fish)	Rank Sum (Three fish)	U	Z	p-level	Z	p-level	Valid N	Valid N	2*1sided exact p
Wild	1065.0	1710.0	362.0	-3.5	**0.000**	-3.487	**0.000**	37	37	**0.000**
Lab	1490.5	1590.5	710.5	-0.5	0.617	-0.500	0.617	39	39	0.619

3.5. Latency to aggression

There was no difference in time to initiate aggression between the wild and lab strain (Table VIII, Survival, $p = 0.629$, Figure 11). The interaction between strain and treatment was not significant (Survival, $p = 0.957$). There was neither any significant difference in the first time of attack between treatments within the strains (Table VIII, $p > 0.05$, Appendix Fig A 8 and Appendix Fig A 9).

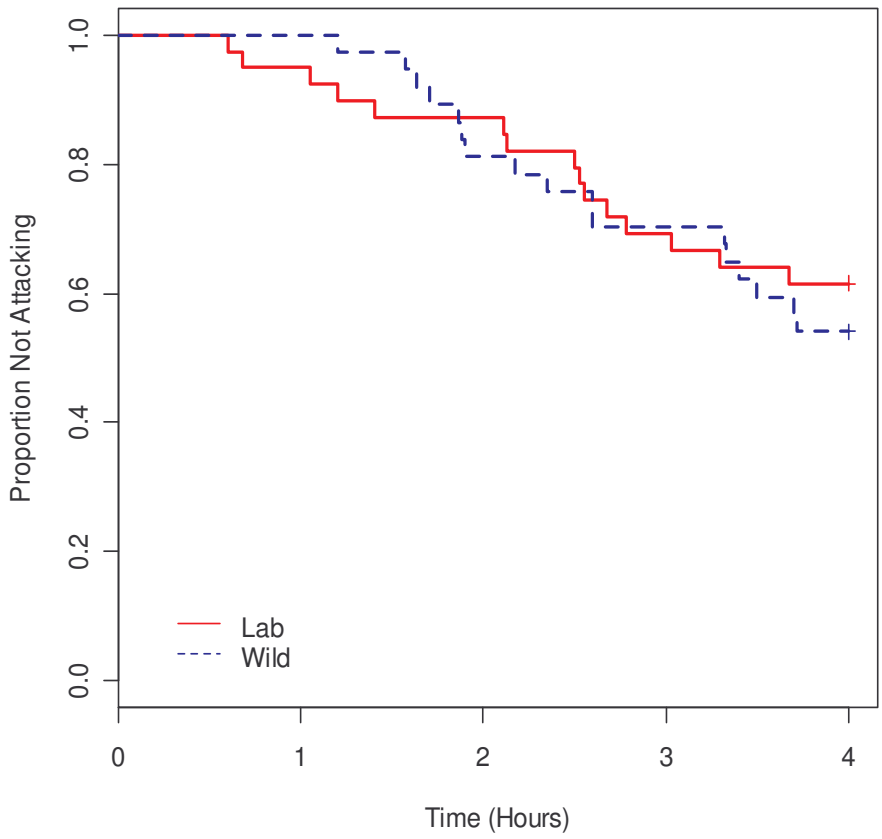

Figure 11. Survival curves showing the latency to aggression for wild and laboratory zebrafish within the first 20 minutes of observation. The figure is based on pooled data from all trials in each strain. The + at the ends of the curves indicate censoring for experiments in which no aggression was made. The blue curve indicates wild fish and red indicates laboratory fish.

Table VIII. Survival analysis comparing the effects of alarm substance and novel object on latency to aggression for both wild and laboratory zebrafish.

General data	Source of Variations	Df	n	Z	p
	Strain	1	76	-0.484	0.629
	Treatment X Strain	68	3		0.957
Wild strain	A Vs A+N	1	19	0.051	0.959
	A Vs C	1	19	0.197	0.844
	A Vs D+N	1	19	0.050	0.96
	C Vs A+N	1	18	0.139	0.89
	A+N Vs D+N	1	18	-0.001	1
	C Vs D+N	1	18	-0.139	0.889
Lab strain	A Vs A+N	1	19	0.526	0.599
	A Vs C	1	19	0.461	0.645
	A Vs D+N	1	19	-0.185	0.854
	C Vs A+N	1	20	-0.061	0.952
	A+N Vs D+N	1	20	-0.719	0.427
	C Vs D+N	1	18	-0.651	0.515

3.6. Frequency of aggression

Laboratory fish attacked not differently (Figure 12) from wild fish (Survival, $p > 0.05$, Table IX). There was no effect of treatment in neither strain ($p > 0.05$, Table IX and Figure 12). The interaction of treatment and strain had no significant effect on the frequency of aggression (GLM, $p > 0.05$).

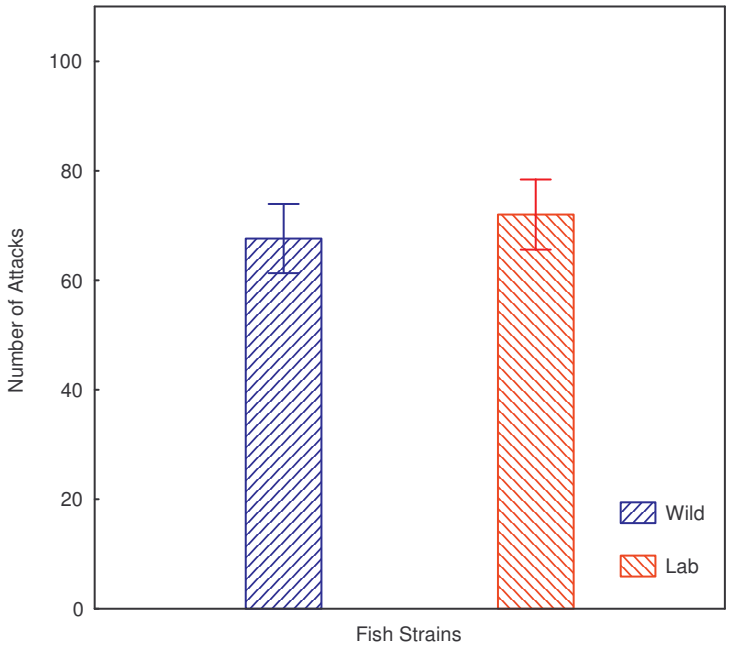

Figure 12. Mean ± SE Number of aggressive attacks within 20 minutes of observation made by wild and laboratory. Figure drawn using pooled data from all trials. Blue bar indicates wild strain and red bar is for laboratory strain.

Table IX. Generalized linear model, comparing the effects of alarm substance and novel object on frequency of aggression of wild and laboratory zebrafish within the first 20 minutes of observation.

Fish Strains	Source of Variations	Df	Residual Df	F	p
	Strain	1	30	0.137	0.714
	Treat X Strain	3	24		0.050
Wild Fish	A Vs A+N	1	7	0.000	0.984
	A Vs C	1	7	0.827	0.394
	A Vs D+N	1	7	0.074	0.794
	C Vs A+N	1	6	0.948	0.368
	A+N Vs D+N	1	6	0.12	0.741
	C Vs D+N	1	6	1.736	0.236
Lab Fish	A Vs A+N	1	5	0.948	0.375
	A Vs C	1	5	0.973	0.369
	A Vs D+N	1	7	3.473	0.105
	C Vs A+N	1	4	3.238	0.146
	A+N Vs D+N	1	6	1.288	0.299
	C Vs D+N	1	6	3.673	0.104

4. DISCUSSION

4.1. Discussion of materials and methods

I tested the response to a novel object and the effect of alarm substance on wild strain (F_1) and the multiple-generation hatchery fish (here after called lab strain) which were available in the laboratory. The genetic homogeneity and age of wild fish could not be ascertained, because they were not born in the laboratory but collected from the same stream and kept together until the experiment. The laboratory strain used were born from the same parents and raised together until tested. Therefore, any result from this study should call for both genetic and environmental explanations. The different sizes and ages of the two strains might also influence the response to alarm substance and novel object. In a study by Dowling and Godin (2002) on killifish (*Fundulus diaphanus*), the time spent in a refuge after exposure to a model trout predator decreased with increasing body size suggesting that small *F. diaphanus* react stronger to predation threat than larger *F. diaphanus*. However, Wright et al. (2003) found that body length did not influence the inspection of novel object (boldness score) and shoaling tendency in zebrafish, although their study did not involve alarm substance. Both strains were sexually mature by the time of study. Since there was limited number of fish and difficulty in separating sexes in the wild strain, mixed sexes were used for all groups. This was also justified by a study of Moretz et al. (2007) that found no difference between sexes of zebra fish in their response to approach a predator dummy.

In this study, all possible trials were conducted; (see section 2.5.1). This made it possible to distinguish between the effect of the alarm substance and novel object and the combined effect of both stimuli on the behaviour, which was an important asset for this study. Further, I studied freezing and aggression as well as approach and shoaling and not only approach and shoaling as Wright et al. (2003) and Wright et al. (2006a). Studies by Moretz et al. (2007) and Snekser et al. (2006) showed that zebrafish prefers to live in shoals but in the present study I also tested shoal preference under a presumed predation threat.

The preparation of alarm substance was standardized for all trials to ensure that there was no variation in concentration due to dilution. The diffusion of alarm substance to the whole tank within one minute was confirmed by testing a blue die and the same rate of diffusion was assumed for alarm substance. Fish were fed twice a day prior to and during the experimental period (but not in the test tank under ongoing experiment) to ensure that their condition did not affect the response to alarm substance. Pfeiffer (1963) stated that fish in poor physical condition show little or no response to alarm substance. He further found that fish which stayed long in aquaria show a stronger response to alarm substance than fish that stayed only a few days and to eliminate this variation, the order of treatments within and between strains were randomized.

Pre-test Trials

The observations from the pre-test trials guided me in the choice of methods. My pre-tests with the lab fish indicated a strong reaction to alarm substance. The choice of three fish per treatment was reached after a behavioural difference was seen when three and five fish were used. In groups of five, fish seemed to school more tightly than fish in groups of three. To avoid bias in assessing the different behaviours, it was easier to observe a group of three than a group of five and was also reducing the number of experimental animals. In the wild, zebrafish form shoals between two-10 fish (Pritchard et al. 2001). Blaser and Gerlai (2006), Krause et al. (1999), Wright et al. (2003) and Wright et al. (2006b) all used single focal fish, but fewer fish than three may have stressed the fish as Snekser et al. (2006) found that zebrafish prefer to join a shoal than to remain in isolation.

My pre-test showed that the responses to novel object and alarm substance took place within the first 20 minutes after presentation of the stimuli with exception of aggression. Thus, I chose to observe and analyze the behaviour responses in detail within the first 20 minutes, while aggression was monitored from initiated attack for 20 minutes within the four hours of the experiment however this could cause over estimation. Based on the pre-tests I determined which behaviour categories to monitor i.e., approach, freezing, shoaling and aggression.

4.2. Discussion of results

In the present study, wild and laboratory zebrafish were subjected to the four different treatment combinations of alarm substance (A), distilled water plus novel object (D+N), alarm substance plus novel object (A+N) and distilled water constituting the control treatment (C). The four behavioural responses analyzed for this discussion were; Novel object approach, freezing, shoaling and aggression. The laboratory strain approached the novel object significantly earlier than the wild strain. Freezing was significantly longer in the lab strain, whereas the wild strain shoaled more. Wild fish but not lab fish preferred to shoal as three than as two fish. The strains did not differ in aggression. Alarm substance had increased shoaling in both strains and freezing in the lab strain and the same tendency in the wild strain.

4.2.1. Approach to novel object

Boldness is associated with exploration of novel object /environment (Wright et al. 2003; Wright et al. 2006a; Wright et al. 2006b). Approach of novel object has been referred to as predator inspection (Pitcher et al. 1986; Wright et al. 2006b). Exploration of potential novel predator carries with it both costs and benefits. A fish approaching a predator may benefit by deterring predators (Magurran 1990; Dugatkin and Godin 1992b), increasing mate attraction (Dugatkin and Godin 1992b; Godin and Dugatkin 1996), acquiring information about identity, location and state of the predator and by visual alarm signalling (Murphy and Pitcher 1997). Costs of approach includes increased risk of mortality, exploitation by shoal members, energetic cost of movement and fin flagging and lost foraging time (Dugatkin and Godin 1992a; Dugatkin and Godin 1992b). To survive and reproduce, prey fish must trade off these costs of predator inspection with the anticipated benefits (Fuiman and Magurran 1994).

Generally, in the present study, the laboratory zebrafish approached the novel object earlier than the wild fish although the two strains showed no difference in the frequency of approach (Figure 2 and Figure 4). This corresponds with my prediction that alarm substance will increase latency to approach novel object strongly in the wild strain. A similar result was found by Wright et al. (2006a). This suggests that lab

strains are bolder (Johnsson and Abrahams 1991; Fernö and Järvi 1998; Fraser et al. 2001; Sneddon 2003; Sundström et al. 2004; Wright et al. 2006a; Brown et al. 2007) than their wild counterparts. Petersson and Järvi (2006) also found that wild brown trout (*S. trutta*) waited longer before entering a predator area compared to the lab fish.

The significant difference in latency to approach novel object between the strains can have the following explanations, in nature zebrafish constantly encounter both known and novel objects in their environments, some of which are potentially dangerous predators. It is known that prey fishes approach potential novel predators upon encounter (Magurran 1986; Pitcher et al. 1986; Godin and Davis 1995; Wright et al. 2003). This would mean that individuals who rush to approach dangerous unfamiliar objects are in great danger of predation. The late approach of novel object by wild strain in the present study might have resulted from selection for cautious approaches after careful assessment of the state and kind of the object encountered. Because predator recognition and the nature of anti-predatory response is finely tuned to the local predation assemblage (Kelley and Magurran 2003), this behaviour is presumably influenced by the environment in zebrafish. The absence of predatory selection in artificial rearing, could result in modification of avoidance behaviour, more specifically reduced anti-predatory response (Alvarez and Nicieza 2003; Wright et al. 2006b)The fact that dangerous predacious objects are absent from the hatchery environments, also means that domesticated lab fish lack experience with dangerous objects and will not always hesitate to inspect a novel object in their environment.

Considering trials with novel object plus distilled water, laboratory fish approached earlier than the wild strain. This might be a result of perceived threat posed by the novel object. Another explanation could base on the activity of the novel object. In the present study the novel object did not move. Murphy and Pitcher (1997) found that European minnows (*Phoxinus phoxinus*) made more inspections towards a live active predator after a strike than before. Kelly and Magurran (2006) suggested that prey may show less cautious response or may stop inspecting the predator if the information they gained in the first approaches suggest that a predator is not actively hunting or pose little threat.

Late approaches to novel object in some trials (Figure 3) may indicate that after stimuli exposure, fish waited for attacks and subsequently decided to explore the novel object. It is possible that the presence of either alarm substance or novel object or both stimuli suppressed the early approach in some treatments which prompted fish to initiate late approaches to assess the state of the novel object. This could be the result of the combined effect of alarm substance and novel object. Also in the combined trial with alarm substance plus novel object, the lab strain showed a significantly earlier approach than the wild strain. It could be that the lab strain was motivated by the smell of alarm substance to explore the novel object or lack of experience with danger associated with novel objects means they have reduced fright response (Alvarez and Nicieza (2003), Johnsson et al. (1996), Robison and Rowland. (2005))

The fact that reaction to alarm substance is observed in both strains with later approaches indicates a strong genetic influence by the alarm substance that is not very plastic even under culture conditions. The response to alarm substance by lab strain could indicate that the selection for alarm substance recognition is so strong that it lies on the instinctive level and gives an unconscious response. However, the lab strain may have been exposed to alarm substance during netting and handling damaging the skin while in the lab and the effect of learning can therefore not be excluded.

4.2.2. Freezing

Freezing is an anti-predatory response exhibited by many prey species (Hall and Suboski 1995; Lozada et al. 2000) and is characterized by complete immobility of the fish over a period of time (Brown and Godin 1999; Bass and Gerlai 2008; Speedie and Gerlai 2008). By freezing, an animal is watching the potential predator while minimizing movement which might attract the predator's attention.

Overall the laboratory strain froze for significantly longer time than did the wild fish (Figure 5). A similar result was found by Fernö and Järvi (1998) with sea ranched *S. trutta* freezing more than the wild strain. Lack of predation selection in the laboratory could explain for the long freezing time in the lab strain. If a prey fish responds to a

real predator by freezing in the wild it could have a greater risk of being caught and subsequently eliminated from the population. In the laboratory with no real predators, freezing in order to hide from a potential predator (the novel object) or a net does not infer any risk since the fish will regardless live to reproduce. Further, compared to other anti-predatory responses like flight and shoaling, freezing is presumably a less costly anti-predatory behaviour which a prey would choose while assessing the motivation of the potential predator.

In the lab strain, fish in trials with alarm substance showed a significantly longer freezing duration than the control group (Figure 6), whereas in the wild strain the alarm substance did not significantly influence freezing, but the same tendency was witnessed. A possible explanation for this could be that since fish has to search for food and do other activities, the perception of alarm substance does not necessarily result in ending a particular activity, but results in appropriate behaviour adjustment depending on the subsequent threat in the vicinity. In the present study introduction of alarm substance was not followed by any threatening cue and this might have reduced reaction to alarm substance in the wild strain. Speedie and Gerlai (2008) stated that under natural conditions, frightened erratic-swimming fishes will freeze if hiding places are available. Though it was not quantified, erratic swimming was observed in both strains upon stimulus presentation in the present study. The wild fish used originated from the streams (Engeszer et al. 2007) and it is possible that the erratic swimming stirs up the debris (especially in shallow slow flowing streams) which provides hiding places for the frightened fish to freeze in the wild. This condition did not exist in the clear test tank and the cue for freezing may thus have been lacking. Fast swimming can also confuse the predator and make it difficult to catch the prey.

In both strains, the novel object did not have any influence on freezing, neither did both stimuli combined had a significant effect. This could be attributed to the inactive nature of the novel object used in this study and similar reasoning as explained under approach of novel object above could explain the lack of influence by the novel object on freezing.

4.2.3. Shoaling

Wright et al. (2006b) defined shoaling as a social assemblage of fish and Pitcher and Parrish (1993) referred to it as fish living together for social reasons. Shoaling plays a key role in mating, foraging and predator avoidance (Heczko and Seghers 1981; Magurran 1990; Pitcher and Parrish 1993) and is affected by changing conditions and differing environments (Wright et al. 2006b).

In the present study wild zebrafish shoaled longer than the lab strain (Figure 7). This may indicate that the anti-predatory function of shoaling is selected for in the wild environments and that the selection pressure is relaxed in the lab. In the laboratory with no predators, shoaling may not be strictly needed and may compete with other activities. Although the same constraints may exist in the wild, the benefits of shoaling i.e. predator detection, mobbing and ganging outweigh the cost. Removal of the need to forage for an extended period of time may result in relaxation of natural selection on traits associated with foraging and predator avoidance in domesticated animals (Wright et al. 2006b). Learning may also be involved. Magurran (1986) found that minnows (*P. phoxinus*) sympatric with pike (*E. lucius)* predator approached the predator model in larger shoals than those which lacked experience with the predator. In the hatchery environment, food is scattered in the tank which means the shoaling does not always increase food search efficiency (Wright et al. 2006b). The proportion of the food an individual can take may in fact be based on its distance from the nearest neighbour; this can further decrease the benefit of shoaling.

For both strains, fish in trials with distilled water plus novel object shoaled significantly longer than fish in the control but were not significantly different from trials with alarm substance and alarm substance plus novel object (Figure 8). It could be that naturally all fish perceive threats posed by every novel object but the intensity of their response is modified by gene selection and or previous experience with such object.

Both strains of fish exposed to alarm substance showed a significantly increased shoaling duration compared to fish in the control (Table VI). A study by Speedie and Gerlai (2008) also found significant effect of alarm substance on increased shoal

cohesion in wild zebrafish. Magurran (1990) stated that, schooling behaviour offers important protection among fishes. The perception of alarm substance might have caused the fish to form a group and start shoaling together in an effort to dilute a possible attack and confuse the predator. An individual in a shoal also gains protection simply through a reduced probability of being the one attack in a shoal (Pitcher and Parrish 1993). Perception of alarm substance seems to induce formation of tight shoals which presents multiple targets to the predator and consequently making the overt predator attack less effective. Shoaling displayed by lab strain could indicate that the behaviour is retained even in the laboratory where predator doesn't exist.

However in the present study both strains exposed to trials with alarm substance plus novel object showed significantly more shoaling than those in the control trials (Table VI). This suggests that both wild and lab fish are affected in a similar way by the presence of alarm substance and novel object; However, because both strains shoaled significantly in trials with novel object but without alarm substance, the effect of novel object could not be eliminated from increased shoaling witnessed in this trial.

An interesting aspect from this study is the decision to shoal as two or three fish. Wild strain shoaled significantly more as three fish than as two whereas there was no difference in the lab strain (Figure 9 and Figure 10). Studies by Moretz et al. (2007) and Snekser et al. (2006) only showed that zebrafish prefers to live in shoals, but in this study I went a step further and tested the shoal preference under a presumed predation threat. In wild when both stimuli were presented simultaneously, shoaling in group of three was elevated significantly. These findings suggest forming shoals is critical for survival in the wild.

4.2.4. Aggression

Aggression in animals could be associated with establishment of dominant hierarchies (Stephan 2007). However the presence of predators and conspecifics affects aggression (Riley et al. 2005).

A number of studies came out with conflicting results comparing aggression in wild and hatchery reared fishes. Whereas Sundström et al. (2003) , Berejikian et al.(2001), Einum and Fleming (1997) and Deverill et al. (1999) found more aggression in hatchery fishes, Mork et al. (1999) and Salonen and Peuhkuri (2004) found more aggression in wild fishes. The present study and those of Dahl et al. (2006), Reinhardt (2001) and Riley et al. (2005) found no effect of hatchery environment on aggression. There was about the same level of aggression in both strains and no difference between treatments. A possible reason for the low level of aggression is that the few numbers of the fish (Three fish per trial) fed to satiation did not necessitate aggression. During my pre-test, trials with more fish (five fish) seem to result in more attacks compared to trials which had three fish. A study by Reinhardt (1999) on Coho salmon and Riley et al.(2005) on steelhead found that the presence of a predator decreased aggression. Mackinlay (2002) pointed out that individual differences, activity level, density and perceived risk from predators affects aggression. In the present study, I observed fish later in some trial chased away tank mates from the novel object, a sign of resource defence. It is therefore possible that, novel objects are not only perceived as predator especially after inspection, but may in fact also provide shelter.

CONCLUSIONS

The results from this study showed that laboratory zebrafish are bolder than the wild strain. Wright et al. (2003) and Wright et al. (2006a) associated boldness with predator inspection. This is supported in the present study by the earlier approach towards the novel object by the lab strain confirming my first prediction. This could mean that the laboratory fish are risk-takers or that they lack experience with predators. The presence of alarm substance did not have a clear influence on latency of approach (Figure 3). This is in contrast to my prediction. I also predicted that alarm substance should increase shoaling and freezing in both strains. My results explicitly confirm this third prediction (Figure 8). Alarm substance increased shoaling in both strains. It also increased freezing in the lab strain with a similar tendency in the wild strain. Shoaling as a reaction to alarm substance could thus be innate. There also seems to be a shift in behaviour from shoaling in the wild to freezing in the lab. Studies by Waldman (1982) found variability in response to alarm substance by zebrafish and Wright et al. (2003), Wright et al. (2006a) and Robison and Rowland (2005) observed great variations in boldness and shoaling tendency among wild strains of zebrafish. Thus, the lab fish I used might have originated from a wild stock with divergent behaviour from the ancestors of the wild fish. The presence of a novel object did not affect freezing in both strains but increased shoaling in both strains in particular in the wild strain (Figure 6 and Figure 8). This partly confirms my fourth prediction. This could suggest that freezing may not be the best anti-predatory response when the predator is within the visual range, thus the fish selected the less risky shoaling behaviour when the novel object was present. This further confirms the boldness in the lab strain that was freezing in the presence of alarm substance. The general pattern of behaviour shown in this study by both strains indicates that both genetic factors and environmental experiences were at play.

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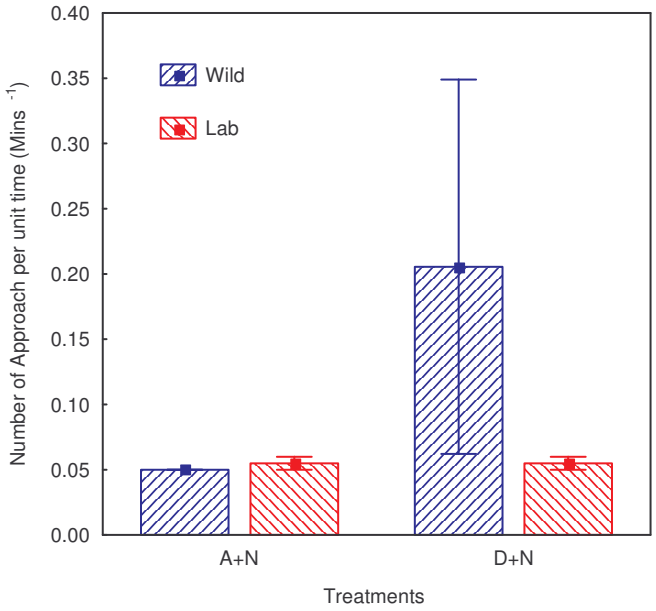
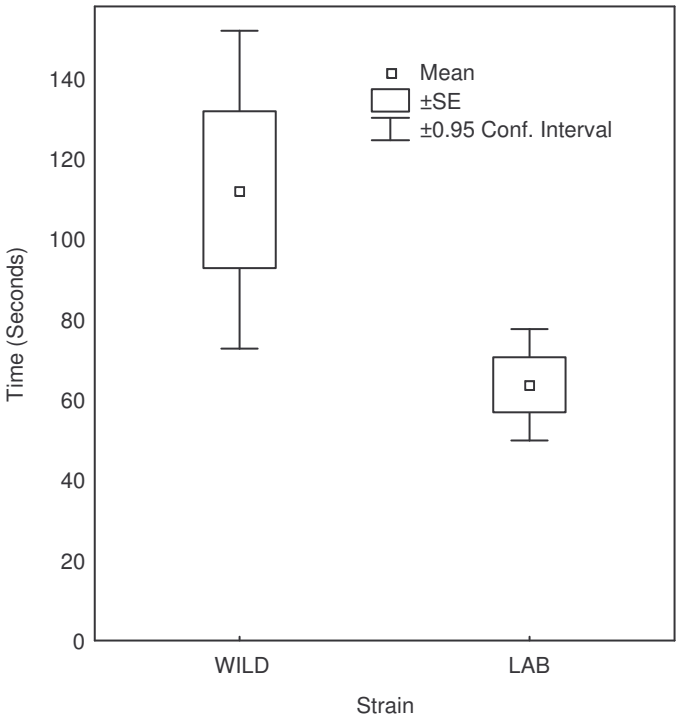
APPENDICES

Appendix A 1. *Frequency of Approaches to Novel Object*

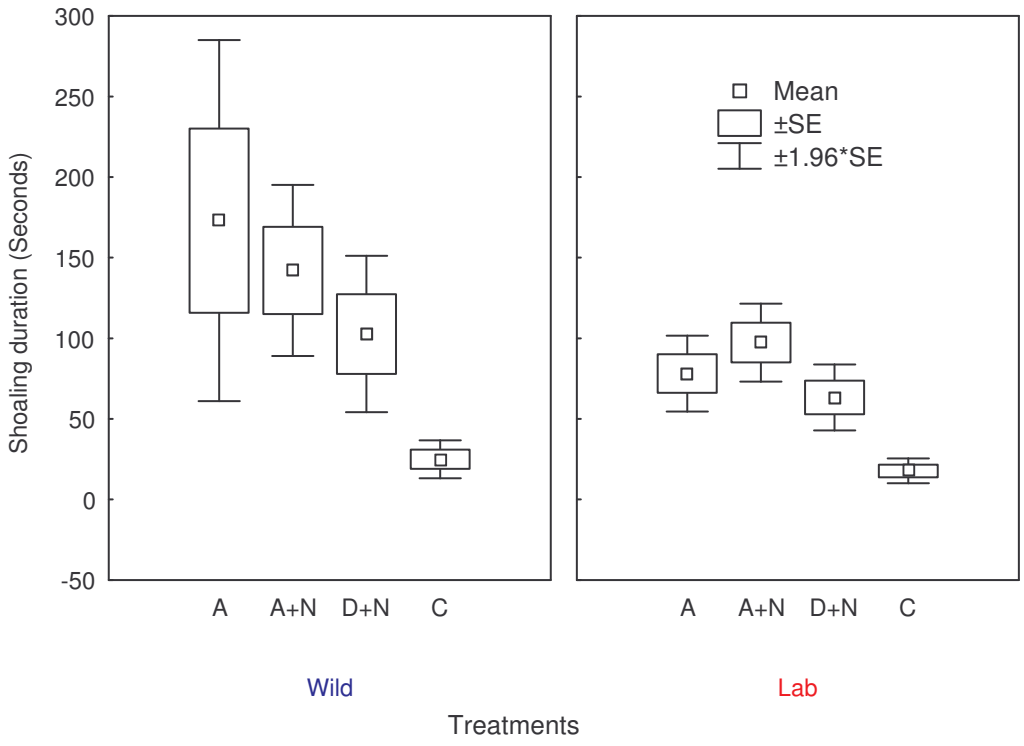


Appendix Fig A 1. Mean ± SE Frequency of approaches for the wild and laboratory strain under different treatments within the first 20 minutes of observation. Blue bars represent wild strain and red bar represents laboratory strain

Appendix A 2.*Two Fish Shoaling Duration*



Appendix Fig A 2. Two fish shoaling duration of wild and laboratory fish subjected to different experimental treatments within the first 20 minutes of observation.

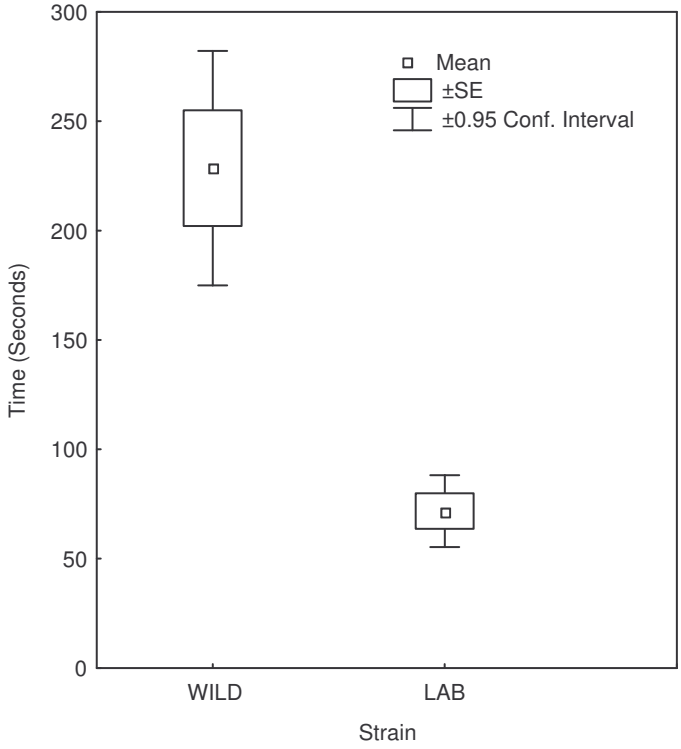


Appendix Fig A 3. Mean ± SE Two fish shoaling duration of wild and laboratory fish subjected to different treatments within the first 20 minutes of observation. A, A+N, D+N, and C has the same meaning as explained in the methods

Appendix Table A 1. ANOVA, Tukey HSD multiple comparisons of means with confidence intervals (95% family-wise confidence level) comparing the effects of alarm substance and novel object on two fish shoal duration of wild and laboratory strains of zebrafish (*Danio rerio*) under different treatments within the first 20 minutes of observation.

Shoal group	**Treatments**	**Difference**	**Lower**	**Upper**	**p adjusted**
			Wild Strain		
Two fish	A Vs A+N	-0.081	-0.993	0.832	0.995
	A Vs C	-1.737	-2.679	-0.794	**0.000**
	A Vs D+N	-0.512	-1.425	0.400	0.437
	C Vs A+N	-1.656	-2.621	-0.691	**0.000**
	A+N Vs D+N	-0.432	-1.368	0.505	0.601
	C Vs D+N	1.224	0.259	2.189	**0.009**
			Lab Strain		
	A Vs A+N	0.0558	-0.662	0.773	0.997
	A Vs C	-1.701	-2.436	-0.966	**0.000**
	A Vs D+N	-0.438	-1.156	0.279	0.364
	C Vs A+N	-1.757	-2.452	-1.062	**0.000**
	A+N Vs D+N	-0.494	-1.170	0.182	0.218
	C Vs D+N	1.263	0.568	1.958	**0.000**

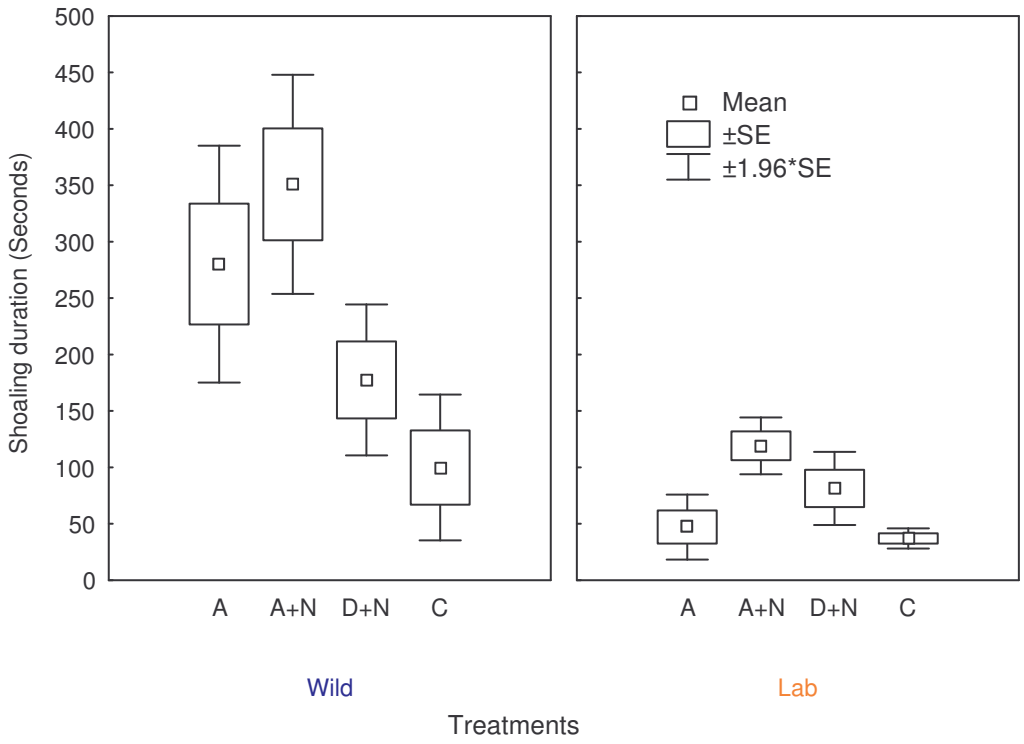
Appendix A 3. *Three Fish Shoaling Duration*



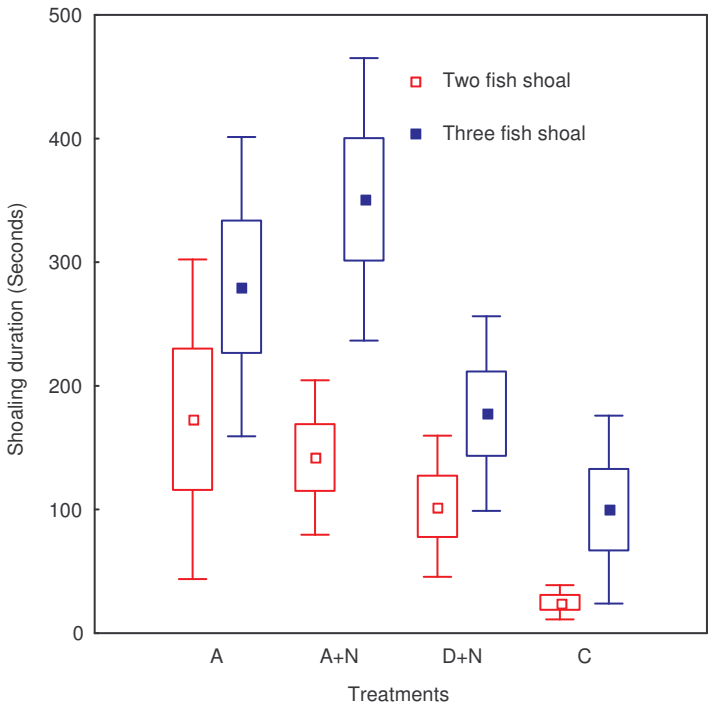
Appendix Fig A 4. Mean ± SE Shoaling duration of three fish within 20 minutes subjected to different treatments.

Appendix Table A 2. ANOVA, Tukey HSD multiple comparisons of means with confidence intervals (95% family-wise confidence level) comparing the effects of alarm substance and novel object on three fish shoal duration of wild and laboratory strains of zebrafish under different treatments within the first 20 minutes of observation.

Shoal group	Treatments	Difference	Lower	Upper	p adjusted
		Wild Strain			
Three fish	A Vs A+N	0.217	-0.705	1.139	0.919
	A Vs C	-1.226	-2.148	-0.304	**0.005**
	A Vs D+N	-0.495	-1.417	0.427	0.477
	C Vs A+N	-1.444	-2.389	-0.497	**0.001**
	A+N Vs D+N	-0.712	-1.658	0.234	0.195
	C Vs D+N	0.731	-0.215	1.677	0.177
		Lab Strain			
	A Vs A+N	0.580	-0.428	1.589	0.415
	A Vs C	-0.623	-1.631	0.386	0.354
	A Vs D+N	-0.101	-1.109	0.907	0.993
	C Vs A+N	-1.203	-2.077	-0.329	**0.004**
	A+N Vs D+N	-0.682	-1.555	0.191	0.169
	C Vs D+N	0.521	-0.352	1.395	0.384



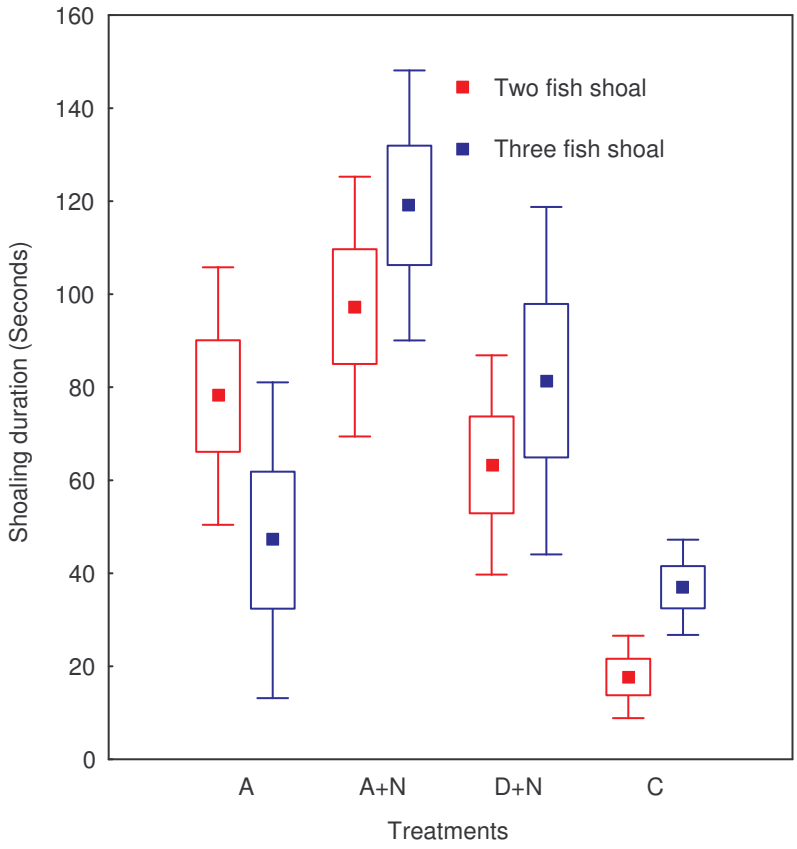
Appendix Fig A 5. Mean ± SE Three fish shoaling duration of wild and laboratory fish subjected to different experimental treatments within the first 20 minutes of observation. A, A+N, D+N and C has the same meaning as explained in the methods.



Appendix Fig A 6. Mean ± SE Two and three fish shoaling duration of wild strain of zebrafish within the first 20 minutes of observation. The blue boxes represent three fish shoals and red boxes are for the two fish shoals.

Appendix Table A 3. ANOVA, Tukey HSD multiple comparisons of means with confidence intervals (95% family-wise confidence level) comparing the effects of alarm substance and novel object on two and three fish shoal duration in wild strain of zebrafish within the first 20 minutes of observation. Marked differences are significant at $p < 0.05$.

	Trials	Shoal group	1	2	3	4	5	6	7	8
1	A	Two fish		0.712	0.884	1.000	1.000	0.402	**0.000**	0.744
2	A	Three fish	0.712		0.088	0.901	0.633	0.999	**0.000**	**0.044**
3	D+N	Two fish	0.884	0.088		0.737	0.950	**0.020**	**0.003**	1.000
4	D+N	Three fish	1.000	0.901	0.737		1.000	0.590	**0.000**	0.557
5	A+N	Two fish	1.000	0.633	0.950	1.000		0.284	**0.000**	0.855
6	A+N	Three fish	0.402	0.999	**0.020**	0.590	0.284		**0.000**	**0.009**
7	C	Two fish	**0.000**	**0.000**	**0.003**	**0.000**	**0.000**	**0.000**		**0.007**
8	C	Three fish	0.744	**0.044**	1.000	0.557	0.855	**0.009**	**0.007**	

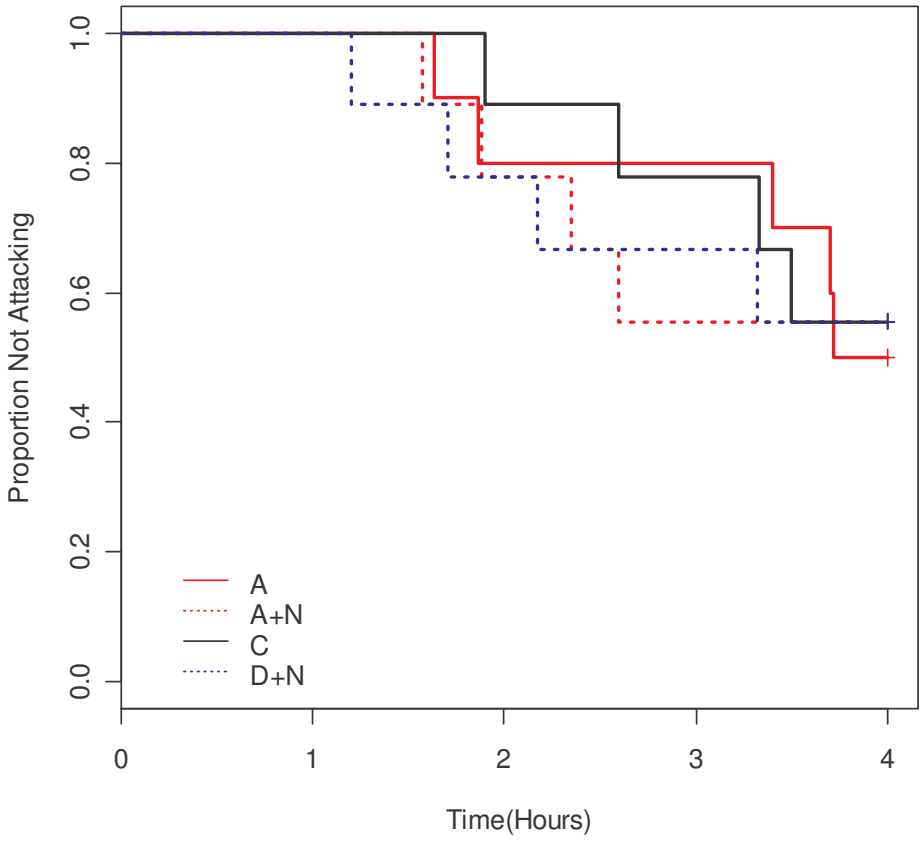


Appendix Fig A 7. Mean ± SE Two and three fish shoaling duration of lab strain of zebrafish within the first 20 minutes of observation. The blue boxes represent three fish shoal and red boxes are for the two fish shoals.

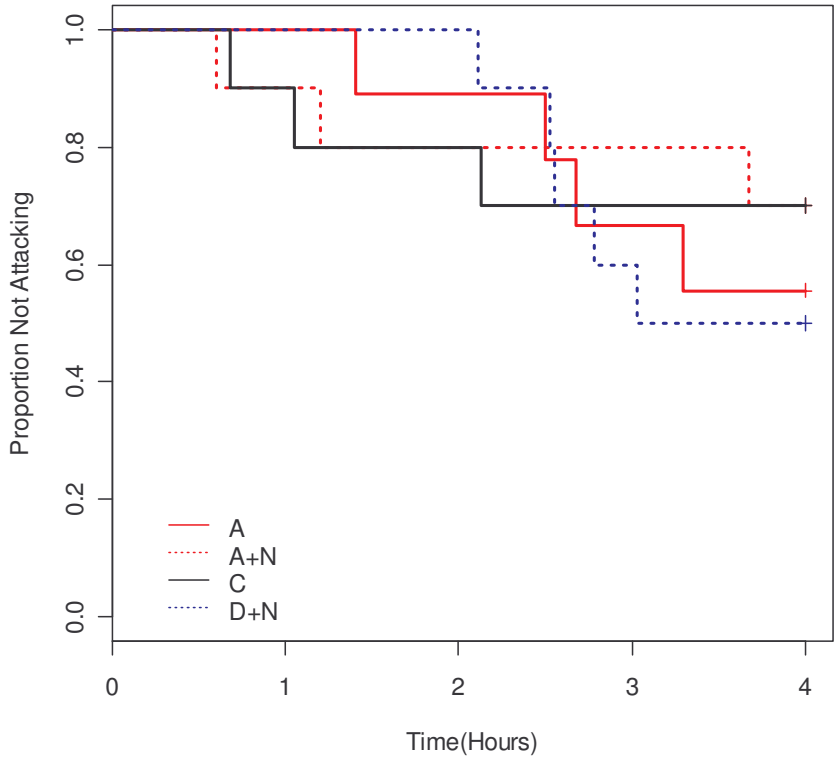
Appendix Table A 4. Tukey HSD multiple comparisons of means with confidence intervals (95% family-wise confidence level) comparing the effects of alarm substance and novel object on two and three fish shoal duration in laboratory strain of zebrafish within the first 20 minutes of observation . Marked differences are significant at $p < 0.05$.

	Trials	Shoal group	1	2	3	4	5	6	7	8
1	A	Two fish		0.318	1	1	0.964	0.815	0.102	0.991
2	A	Three fish	0.318		0.263	0.23	**0.028**	**0.008**	0.999	0.828
3	D+N	Two fish	1	0.263		1	0.973	0.832	0.052	0.975
4	D+N	Three fish	1	0.232	1		0.982	0.864	**0.044**	0.964
5	A+N	Two fish	0.964	**0.028**	0.973	0.982		1.000	**0.003**	0.500
6	A+N	Three fish	0.815	**0.008**	0.832	0.864	1.000		**0.001**	0.243
7	C	Two fish	0.102	0.999	0.052	**0.044**	**0.003**	**0.001**		0.411
8	C	Three fish	0.991	0.828	0.975	0.964	0.500	0.243	0.411	

Appendix A 4. *Latency to Aggression*



Appendix Fig A 8. Survival curves showing latency to aggression for the wild strain within the first 20 minutes of observation. The + at the ends of the curves indicate censoring for experiments in which no aggression was made. The red curve indicates which contained alarm substance.



Appendix Fig A 9**.** Survival curves showing latency to aggression for the lab strain within the first 20 minutes of observation. The + at the ends of the curves indicate censoring for experiments in which no aggression was made. The red curve indicates which contained alarm substance.

Appendix A 5. *Frequency of Aggression*

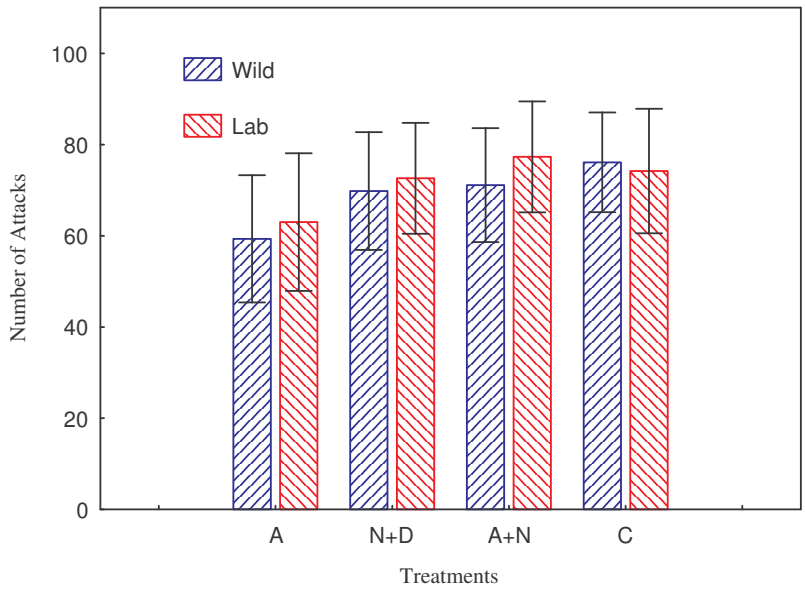


Figure 13. Mean ± SE Number of aggressive attacks by wild and laboratory *Danio rerio* within the first 20 minutes of observation.

Table of data used for the different behaviour measures.

Latency to approach novel object				Frequency to novel object	
Strains	Treatments	Time	Censoring status	Counts	Counts/minutes
Wild	A+N	986	1	1	0.05
Wild	A+N	1200	0	1	0.05
Wild	A+N	1200	0	1	0.05
Wild	A+N	1200	0	1	0.05
Wild	A+N	499.2	1	1	0.05
Wild	A+N	62	1	1	0.05
Wild	A+N	435.2	1	1	0.05
Wild	A+N	514	1	1	0.05
Wild	A+N	1200	0	1	0.05
Wild	D+N	1200	0	0	0
Wild	D+N	1200	0	1	0.05
Wild	D+N	1020	1	1	0.05
Wild	D+N	1158	1	2	0.1
Wild	D+N	1200	0	1	0.05
Wild	D+N	1200	0	1	0.05
Wild	D+N	1200	0	27	1.35
Wild	D+N	273.84	1	2	0.1
Wild	D+N	94.8	1	2	0.1
Lab	A+N	305.32	1	2	0.1
Lab	A+N	43	1	1	0.05
Lab	A+N	71	1	1	0.05
Lab	A+N	62	1	1	0.05
Lab	A+N	47	1	1	0.05
Lab	A+N	1200	0	1	0.05
Lab	A+N	701	1	1	0.05
Lab	A+N	1200	0	1	0.05
Lab	A+N	82	1	1	0.05
Lab	A+N	53	1	1	0.05
Lab	D+N	892	1	1	0.05
Lab	D+N	53	1	1	0.05
Lab	D+N	85	1	1	0.05
Lab	D+N	954	1	2	0.1
Lab	D+N	1200	0	1	0.05
Lab	D+N	974.92	1	1	0.05
Lab	D+N	72	1	1	0.05
Lab	D+N	23	1	1	0.05
Lab	D+N	1.56	1	1	0.05
Lab	D+N	901	1	1	0.05

Latency and Frequency of Aggression				
Strains	Treatments	Counts	Latency/ Time	Status
WILD	A	8	3.72	1
WILD	A	58	1.63	1
WILD	A	24	3.4	1
WILD	A	NA	4	0
WILD	A	27	1.86	1
WILD	A	NA	4	0
WILD	A	NA	4	0
WILD	A	NA	4	0
WILD	A	13	3.7	1
WILD	A	NA	4	0
WILD	A+N	NA	4	0
WILD	A+N	NA	4	0
WILD	A+N	32	1.88	1
WILD	A+N	NA	4	0
WILD	A+N	16	1.57	1
WILD	A+N	44	2.35	1
WILD	A+N	NA	4	0
WILD	A+N	NA	4	0
WILD	A+N	13	2.6	1
WILD	D+N	29	1.2	1
WILD	D+N	12	2.17	1
WILD	D+N	NA	4	0
WILD	D+N	NA	4	0
WILD	D+N	14	1.7	1
WILD	D+N	NA	4	0
WILD	D+N	37	3.32	1
WILD	D+N	NA	4	0
WILD	D+N	NA	4	0
WILD	C	13	3.5	1
WILD	C	NA	4	0
WILD	C	28	2.6	1
WILD	C	NA	4	0
WILD	C	NA	4	0
WILD	C	NA	4	0
WILD	C	NA	4	0
WILD	C	57	1.9	1
WILD	C	57	3.33	1
LAB	A	22	2.68	1
LAB	A	20	2.5	1
LAB	A	NA	4	0
LAB	A	13	1.4	1
LAB	A	NA	4	0
LAB	A	7	3.3	1
LAB	A	NA	4	0
LAB	A	NA	4	0
LAB	A	NA	4	0
LAB	A+N	NA	4	0
LAB	A+N	NA	4	0
LAB	A+N	NA	4	0
LAB	A+N	NA	4	0
LAB	A+N	28	1.2	1

LAB	A+N	NA		4	0
LAB	A+N	NA		4	0
LAB	A+N		29	0.6	1
LAB	A+N	NA		4	0
LAB	A+N		9	3.68	1
LAB	D+N	NA		4	0
LAB	D+N		18	2.55	1
LAB	D+N	NA		4	0
LAB	D+N	NA		4	0
LAB	D+N		4	3.03	1
LAB	D+N	NA		4	0
LAB	D+N		37	2.11	1
LAB	D+N	NA		4	0
LAB	D+N		89	2.78	1
LAB	D+N		73	2.53	1
LAB	C	NA		4	0
LAB	C	NA		4	0
LAB	C	NA		4	0
LAB	C	NA		4	0
LAB	C	NA		4	0
LAB	C	NA		4	0
LAB	C		10	1.05	1
LAB	C		12	2.13	1
LAB	C		13	0.68	1
LAB	C	NA		4	0

Freezing duration		
Strains	Treatments	Time
WILD	A	71
WILD	A	0
WILD	A	57
WILD	A	9
WILD	A	0
WILD	A	4
WILD	A	0
WILD	A	13
WILD	A	134
WILD	A	50
WILD	A+N	0
WILD	A+N	0
WILD	A+N	0
WILD	A+N	0
WILD	A+N	0
WILD	A+N	341
WILD	A+N	0
WILD	A+N	0
WILD	A+N	0
WILD	D+N	0
WILD	D+N	5
WILD	D+N	0
WILD	D+N	0
WILD	D+N	0
WILD	D+N	0
WILD	D+N	0
WILD	D+N	74
WILD	D+N	31
WILD	C	0
WILD	C	64
WILD	C	0
WILD	C	0
WILD	C	3
WILD	C	0
WILD	C	0
WILD	C	0
WILD	C	0

Freezing duration		
Strains	Treatments	Time
LAB	A	240
LAB	A	398
LAB	A	73
LAB	A	108
LAB	A	380
LAB	A	54
LAB	A	360
LAB	A	82
LAB	A	0
LAB	A	8
LAB	A+N	20
LAB	A+N	34
LAB	A+N	18
LAB	A+N	380
LAB	A+N	0
LAB	A+N	74
LAB	A+N	214
LAB	A+N	259
LAB	A+N	18
LAB	A+N	79
LAB	D+N	9
LAB	D+N	36
LAB	D+N	84
LAB	D+N	0
LAB	D+N	19
LAB	D+N	8
LAB	D+N	48
LAB	D+N	10
LAB	D+N	151
LAB	D+N	26
LAB	C	6
LAB	C	0
LAB	C	21
LAB	C	43
LAB	C	117
LAB	C	14
LAB	C	17
LAB	C	12
LAB	C	10

Shoaling duration				
Strains	Treatments	Two fish	Three fish	Total
WILD	A	85	641	726
WILD	A	126	285	411
WILD	A	98	447	545
WILD	A	69	163	232
WILD	A	103	108	211
WILD	A	76	240	316
WILD	A	279	283	562
WILD	A	658	119	777
WILD	A	135	376	511
WILD	A	100.9	140	240.9
WILD	A+N	205	407	612
WILD	A+N	167	346	513
WILD	A+N	308	511	819
WILD	A+N	111	461	572
WILD	A+N	84	392	476
WILD	A+N	118	182	300
WILD	A+N	90.1	47.52	137.62
WILD	A+N	165	467	632
WILD	A+N	30.5	344	374.5
WILD	D+N	63.73	92	155.73
WILD	D+N	163	168	331
WILD	D+N	47	63	110
WILD	D+N	26	277	303
WILD	D+N	113	226	339
WILD	D+N	24	217	241
WILD	D+N	76	165	241
WILD	D+N	175	351	526
WILD	D+N	236	38.79	274.79
WILD	C	47	340	387
WILD	C	20.72	64	84.72
WILD	C	0	76	76
WILD	C	5	17	22
WILD	C	19	33	52
WILD	C	39.6	63	102.6
WILD	C	52	40	92
WILD	C	16	124	140
WILD	C	25.3	142	167.3
LAB	A	73	87	160
LAB	A	78.73	106	184.73
LAB	A	75	59	134
LAB	A	65.71	0	65.71
LAB	A	74.55	0	74.55
LAB	A	0	0	0
LAB	A	118	32	150
LAB	A	91.97	35	126.97
LAB	A	126	105	231
LAB	A+N	102.3	118	220.3
LAB	A+N	92	117	209
LAB	A+N	84	73	157
LAB	A+N	44	91	135
LAB	A+N	59	65	124
LAB	A+N	181	105	286

LAB	A+N	109.32	152	261.32
LAB	A+N	66	197	263
LAB	A+N	108	114	222
LAB	A+N	127.71	159	286.71
LAB	D+N	45	134	179
LAB	D+N	18	3	21
LAB	D+N	83	117	200
LAB	D+N	81	36	117
LAB	D+N	27	177	204
LAB	D+N	49	40	89
LAB	D+N	134	56	190
LAB	D+N	73	102	175
LAB	D+N	67	63	130
LAB	D+N	56	86	142
LAB	C	8	52	60
LAB	C	0	36	36
LAB	C	8	57	65
LAB	C	25	55	80
LAB	C	32	30	62
LAB	C	3	27	30
LAB	C	20	30	50
LAB	C	36.72	33	69.72
LAB	C	23	39	62
LAB	C	21.36	11	32.36

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