

Disturbance cues in prey fishes: an additional source of chemosensory risk assessment

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## **Abstract**

Disturbance cues in prey fishes: an additional source of chemosensory risk assessment

Meaghan Vavrek

Chemosensory cues, including disturbance cues, are important indicators of predation risk in aquatic systems. To date, little is known about disturbance cues although they are thought to exist in a variety of fishes and are believed to indicate lower risk as compared to damage-released alarm cues. Previous studies suggest disturbance cues are composed of a metabolic waste product, likely ammonium, though this has not been directly tested in fish.

For my thesis, I wanted to verify the presence of and characterize the response to disturbance cues in two taxonomically distant species: juvenile convict cichlids (*Archocentrus nigrofasciatus*) and juvenile rainbow trout (*Oncorhynchus mykiss*). I also wanted to test the assumption that the disturbance cue is a metabolic waste product, specifically ammonium.

Both species responded to the disturbance cue in a threat-sensitive manner, which was expected given the low risk nature of the cue. As well, my results suggest ammonium is not likely the major component of the disturbance cue although it may be some type of metabolic waste product. Future work is needed in order to determine the major constituent of the disturbance cue and to further examine its ecological importance.

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# **Chapter 1: Predator avoidance in fishes is facilitated by chemosensory cues.**

## **Introduction:**

The predator avoidance patterns of most (if not all) prey species are shaped by a series of cost-benefit trade-offs. Clearly, the early detection and avoidance of predators confers increased survival benefits to individual prey. However, time spent trying to evade a predator means that less energy is available for other fitness related activities such as mating, foraging, and territorial defence (Godin and Smith, 1988; Lima and Dill, 1990). Therefore, in order to optimize fitness, prey individuals should respond in an appropriate manner to the current level of risk; showing a reduction in anti-predator behaviour in response to low levels of risk and a graded increase to higher threat levels (Helfman, 1989; Lima and Dill, 1990; Foam et al., 2005). Such a trade-off is referred to as threat-sensitivity (Helfman, 1989) and the ability to make threat-sensitive behavioural decisions assumes that prey can reliably assess local predation threats (Brown et al. 2006).

In fishes, visual and chemical cues are the most well studied sensory modalities (Chivers and Smith, 1998; Kats and Dill, 1998) although they likely confer different levels of risk and information (Brown, 2003). Visual cues are spatially and temporally more reliable than chemical cues but are risky as they increase the likelihood that prey will also be seen by the predator (Kats and Dill, 1998). As well, visual cues such as a predator's body position and/or behaviour can be manipulated by predators (Murphy and Pitcher, 1997) whereas chemical cues are less likely to be manipulated and may therefore

give a more honest signal (Brown and Magnavacca, 2003). Chemical cues are also likely to be more useful in highly turbid waters, low light conditions and in highly complex environments; conditions where visual cues may be of more limited value. In these instances, chemical cues are probably of primary importance in the early detection and avoidance of predators (Kats and Dill, 1998; Bryer et al., 2001). Examples of such cues used for predator detection and local risk assessment are found in a wide variety of fishes, including Cichlidae and Salmonidae (Brown and Smith, 1997; Chivers and Smith, 1998; Brown et al., 2006).

Chemosensory cues used for predator detection are released by prey at different points during the predation sequence (Bryer et al., 2001). For example, a cue released when prey have been disturbed, but not damaged (likely before an attack) is referred to as a disturbance cue (Chivers and Smith, 1998). During an attack, damage-released alarm cues may be released from the epidermis of fishes (Chivers and Smith, 1998) and after an attack many fish retain recognition of predator odours. All three cues have been shown to increase anti-predator behaviour and confer an increased survival benefit (Chivers and Smith, 1998; Mirza and Chivers, 2002; Darwish et al. 2005). However, damage released alarm cues and predator odours have received far more study.

#### Damage-released alarm cues:

There have been a large number of studies examining the chemistry and function of damage-released alarm cues over the past 15 years, focusing on a wide range of taxonomically diverse prey species, predominantly in fishes (Chivers and Smith, 1998). In general, damage-released alarm cues are found in the epidermis (Chivers and Smith,

1998) and are released only after the prey has been injured and their skin has been mechanically damaged. This often occurs during an attack by a predator, making it a reliable signal to the receiver fish (Chivers et al., 2007). As they are reliable signals, alarm cues can elicit strong species-typical anti-predator responses such as a decrease in movement and foraging (Chivers and Smith, 1998). Such obvious anti-predator reactions are termed overt responses (Smith, 1999). However, fish can also respond in covert ways where no immediately measurable behaviour may be detected but long term effects such as life-history changes or learning occur (Brown and Smith, 1996; Brown et al., 2004a; Chivers et al., in press). This can also lead to increased probability of escape from a predator (Mirza and Chivers, 2003) and altered timing of nest emergence (Mirza et al., 2001). It has been suggested that by not responding overtly, prey can conserve energy for other important activities, such as foraging and finding mates (Foam et al., 2005). The extent to which prey respond will depend on the level of perceived risk (Helfman, 1989; Foam et al., 1995; Brown et al., 2006).

#### Predator Odour:

Fishes can also chemically assess local predation risk through the use of the odours of predators themselves. This recognition may be innate (Berejikian et al., 2003) but in many cases is learned. Such an example is illustrated in pike-naïve fathead minnows (*Pimephales promelas*) that can learn to associate pike odour as dangerous via pairing of the odour with conspecific alarm cue (Mathis and Smith, 1993). Many fish can also 'label' a predator as such if its diet includes conspecifics, although this has been suggested to be due to a recognition of alarm cue in the gut rather than the odour of

predators themselves (Mathis and Smith, 1993; Brown et al., 1995a; Brown et al., 1995b).

Disturbance cues:

Much less is known about disturbance cues, which are released by uninjured fish in the presence of a predator (Bryers et al., 2001) and have been found to exist in various aquatic organisms including: crayfish, *Oronectes virilis*; hermit crabs, *Calcinus laevimanus*; larval red-legged frogs, *Rana aurora*; convict cichlids, *Archocentrus nigrofasciatus*; Iowa darters, *Etheostoma exile*; slimy sculpins, *Cottus cognatus*; pacus *Piaractus mesopotamicus*; brook charr, *Salvelinus fontinalis* and potentially rainbow trout, *Oncorhynchus mykiss* (Hazlett, 1990a; Hazlett, 1990b; Wisenden et al., 1995; Kiesecker et al., 1999; Lebedeva et al., 1999; Jordao and Volpato, 2000; Bryer et al., 2001; Mirza and Chivers, 2002; Jordao, 2004).

Disturbance cues are not necessarily released intentionally, and may be the same chemical that is released in stressed fish (Wisenden et al., 1995; Kiesecker et al., 1999; Jordao, 2004). Stressed fish have an increased metabolism, suggesting that the disturbance cue could be a metabolic waste product, such as ammonium ( $\text{NH}_4^+$ ), released in the urine or across the gills (Hazlett, 1990a; Kiesecker et al., 1999). Further evidence the cue is a metabolic waste product is that it can be exhausted; Iowa darters that were pre-stressed failed to cause a response to the disturbance cue in conspecific receivers (Chivers and Smith, 1998).

Since disturbance cues should warn prey of a threat before an attack occurs, there should be an increased chance of survival during an attack. Mirza and Chivers (2002)

showed that brook charr survived longer in the presence of a predator if they had previously been exposed to the disturbance cue and the predator chemical odour. This suggests that there is a fitness benefit to detecting and responding to disturbance cues from conspecifics.

All three chemosensory cues are likely to confer different levels of threat. Since the alarm cue causes actual damage to the donor fish, it is thought that it signifies a greater risk than that of the disturbance cue or of predator odour (Wisenden et al., 1995; Chivers and Smith, 1998; Bryer et al., 2001; Jordao, 2004; Ferrari et al., 2005). In regards to the disturbance cue in particular, previous studies in fish suggest that it may also result in less overt behaviour (Wisenden et al., 1995; Jordao and Volpato, 2000; Bryer et al., 2001; Jordao, 2004).

To date, in comparison to the chemical alarm and predator odour cues, little is known about disturbance cues and their potential role in predator avoidance. In order to have a better understanding of these cues, I performed a series of experiments. First, I wanted to verify the existence of disturbance cues in juvenile convict cichlids and juvenile rainbow trout. My second goal was to characterize the behavioural response to these cues. Since the disturbance cue is thought to signify lower risk as compared to the alarm cue (Wisenden et al., 1995; Jordao and Volpato, 2000; Bryer et al., 2001; Jordao, 2004), I expected to see a less intense, graded response. Such threat-sensitive behaviour is observed in cichlids and trout when perceived risk is altered; in small groups, both fishes respond in an all-or-none “hypersensitive” manner to the alarm cue (Mirza and Chivers, 2003; Brown et al., 2006). However, as group size increases, inherent risk decreases and the anti-predator response is graded (Brown et al., 2006).

The third goal of my thesis was to examine the hypothesis that these cues are composed of a metabolic waste product such as ammonium released by stressed individuals. Most studies suggest the probable cue is ammonium based on work done on crayfish and red-legged frogs (Hazlett, 1990a; Kiesecker et al., 1999). However, this hypothesis has not been directly investigated in fish (Wisenden et al., 1995; Jordao and Volpato, 2000; Bryer et al., 2001; Jordao, 2004). I wanted to test this assumption by measuring the change of ammonium concentration in disturbed fish and by doing behavioural assays when given ammonium directly. I expected to find an increase in ammonium concentration in water from disturbed tanks and an elevated anti-predator response in fish receiving ammonium. Finally, I wanted to determine whether the cue is conserved across taxonomically distant species. If the disturbance cue is a metabolic waste product, it should be the same cue for all fish that also excrete the same type of waste. Therefore prey should respond to heterospecific cues (Hazlett, 1990a). This is contrary to the alarm cue, which is conserved taxonomically in the absence of learning (Wisenden and Sargent, 1997; Mirza and Chivers, 2001; Brown et al., 2003).

## **Chapter 2: The presence of a graded response pattern to a disturbance cue in juvenile convict cichlids and rainbow trout.**

### **Introduction:**

Previous work suggests that disturbance cues evoke subtle anti-predator behavioural responses (Wisenden et al., 1995; Jordao and Volpato, 2000; Bryer et al., 2001; Jordao, 2004) and exist in a variety of species, including convict cichlids and rainbow trout (Lebedeva et al., 1999; Jordao, 2004). However, of the few studies that exist none have attempted to characterize the response at varying concentrations in order to determine whether a threat sensitive response is present.

To date, few studies have examined the response to disturbance cues in prey fishes (see Introduction). In convict cichlids, the only study done measured the behavioural response of one cichlid receiving a number of cues (over several days, one which included the disturbance cue) from a single donor (Jordao, 2004). The donor and receiver were in separate, visually isolated tanks but were connected so that water could pass from the donor to the receiver via gravity flow. Her experimental design set-up was thus limited: it did not allow same-day comparisons of the disturbance cue to an undisturbed control; did not rule out the possibility that the fish were communicating via auditory means (Wisenden et al., 1995); and most importantly, did not look for changes in behaviour in response to varying amounts of cue. As this cue is believed to evoke more subtle responses in comparison to the alarm cue (Wisenden et al., 1995; Jordao and Volpato, 2000; Bryer et al., 2001) it is not surprising that she found very little response

except for an increase in movement, which she attributed to predator inspection behaviour.

While no behavioural studies have examined the role of disturbance cues in rainbow trout, Mirza and Chivers (2002) reported the existence of a disturbance cue in brook charr, which are closely related to rainbow trout. As well, a physiological study suggests that rainbow trout themselves may produce a disturbance cue (Lebedeva et al., 1999). In their study, Lebedeva et al. (1999) exposed trout to an electric current (which they considered a stress) and then collected the exometabolites to give to receiver fish. They found changes in physiological (increased gill ventilation frequency) and biochemical (increased glucose concentration) parameters in receiver fish that they suggested were a response to 'chemical alarm substances' released in the urine or excrement of stressed trout.

The goal of this chapter was to verify the existence of and characterize the response to a disturbance cue in juvenile convict cichlids and juvenile rainbow trout. I tested for the presence of disturbance cues in receiver fish by giving them water from disturbed and undisturbed conspecific donors. In cichlids, I introduced low (3 and 5 donors), intermediate (7 and 10 donors), and high (12 and 15 donors) levels of cue to receiver fish. In trout, I used 5 and 10 donors only. Donor numbers were chosen randomly in order to determine a suitable group size for future experiments. I then compared the anti-predatory behaviour before and after introduction of the stimulus.

I predicted that there would be a measurable, graded increase in anti-predatory behaviour to varying concentrations of the disturbance cue; low levels of cue should signify low risk and therefore correspond with a small change in anti-predatory behaviour

(if any). High levels of cue should indicate a higher threat level, so I expected to see more intense anti-predator behaviour. An underlying assumption of my experiment was that a greater number of donor fish would produce a greater amount of disturbance cue. Such assumptions may not always be true, so I performed a second experiment in which I maintained the donor number (10 for cichlids and 5 for trout) and made various dilutions of disturbance cue (100, 75, 50, 25 and 0%). Again, I predicted both species to show a graded response to the disturbance cue. A graded response could consist of a steady increase in anti-predatory behaviour at higher concentrations of disturbance cue, a change in one type of anti-predator behaviour while others remain the same, or both (Brown et al., 2006).

### **General Methods:**

#### Test fish:

##### *Cichlids:*

Juvenile convict cichlids from the same stock population were used as disturbance cue donors (mean  $\pm$  SD = 4.00  $\pm$  1.25 g; 4.40  $\pm$  0.57 cm standard length, SL: the distance from the most anterior part of the head to the distal end of the last whole vertebral centrum) and receivers (2.22  $\pm$  1.07 g; 3.46  $\pm$  0.67 cm SL) and were only used once per experiment. The stock population consisted of descendants of lab bred crosses between store bought and wild caught fish from Costa Rica. All test fish were held in visually isolated tanks containing a gravel substrate, dechlorinated tap water maintained at  $\sim$ 28°C, and at a 12:12 hour light:dark cycle for at least one day prior to testing.

Donor tanks were 20 L and contained a running filter that was turned off one hour before testing. I did this to ensure that the cue, which is thought to be a nitrogenous waste product such as ammonium (Hazlett, 1990a; Kiesecker et al., 1999), would not be removed by the filter.

Receiver fish were arbitrarily chosen and matched for size before being placed in 37 L tanks; each of which had a single air stone and an injection tube affixed near the back. I paired receiver fish since singleton cichlids do not show threat-sensitive response patterns (Brown et al., 2006). Four equidistant lines on the fronts of the tanks were drawn using a black grease pencil in order to make area use measurements.

*Trout:*

Juvenile rainbow trout were obtained from “Pisciculture des Arpents Verts” in Ste. Edwidge de Clifton, Quebec. All trout were from the same population and were used as disturbance cue donors ( $1.10 \pm 0.47$  g;  $3.69 \pm 0.35$  cm SL) and receivers ( $1.07 \pm 0.37$  g;  $3.86 \pm 0.35$  cm SL) once per experiment. All test fish were held in identical conditions as described above for the cichlids, with the exceptions that the water temperature was kept at  $\sim 18^{\circ}\text{C}$ , only one receiver fish was added to each 37 L tank and three equidistant lines were drawn on the fronts of receiver tanks.

Collection of disturbance cue:

In order to generate the disturbance cue, I slowly passed a model predator (a wooden fish lure 12 cm long and approximately 2.5 cm wide attached to a 32.5 cm long glass rod) back and forth along the length of a donor tank containing no running filter. Both cichlids and trout reduced movement and form tighter shoals near the bottom corner of their respective tanks when the model predator was introduced (personal observations). The

model was passed 20 times, taking care to avoid touching the fish and inadvertently releasing the damage-released alarm cue. After one minute, I took a sample of the water from the disturbed tank and from a visually isolated undisturbed control tank.

### **Experiment 1: Establishing the presence of a disturbance cue**

#### *Cichlids:*

Test fish were fed commercial flake food *ad libitum* until one hour prior to testing. Each trial lasted for 10 minutes, consisting of a five minute pre-stimulus and a five minute post-stimulus period. The stimulus consisted of 10 mL of water from disturbed or undisturbed donors from various group sizes: three, five, seven, ten, twelve and fifteen donors. Donor numbers were chosen randomly and were varied in order to determine a suitable group size for future experiments. Prior to adding the stimulus, I withdrew and discarded 60 mL of water from the injection tube to remove any stagnant water. Trials were videotaped and individual scores were averaged per tank.

I compared the anti-predator behaviour of fish during the post-stimulus period to behaviour in the pre-stimulus period for each behavioural category (post-pre). The behavioural categories I took included mean area use (body position on a four grid map that was drawn on the front of the tank; a score of 1 corresponds with the bottom quarter of the tank and 4 the top quarter), total time spent moving (seconds) and the foraging rate (average number of foraging attempts per minute). In order to quantify foraging behaviour, I added 5 mL of frozen brine shrimp in suspension immediately prior to each measurement period. A decrease of any of these behavioural measures signifies an anti-predator response in juvenile convict cichlids (Wisenden and Sargent, 1997; Brown et al.,

2004b). All treatments were repeated 15 times, with the exception of the 15 donor treatment: 14 disturbed and 13 undisturbed treatments were used due to poor videotape visibility in three tanks.

Since I had large sample sizes, I used parametric tests throughout. I first performed a MANOVA followed by three 2-way ANOVAs for each individual behaviour measure. Undisturbed donors appeared to evoke a response for foraging rate and so data pre and post stimulus were analyzed using a repeated measures ANOVA. The means  $\pm$  the standard error (SE) were used to graph all my results. All statistics were performed using SPSS 10.1.

*Trout:*

Methods were repeated on trout using trout donors and receivers and were the same as mentioned above, with a few exceptions. First, I used five and ten donors from disturbed or undisturbed conspecifics as my treatment groups. I only used two donor group sizes for trout since I was better able to predict suitable sizes based on the previous experiments done in cichlids. Second, test fish were fed pellets *ad libitum* until one hour prior to testing. Third, measurements of anti-predator behaviour taken included time spent moving (seconds), number of foraging attempts (reported as foraging rate), and time spent on the bottom (seconds spent in the bottom third of the tank). An anti-predator response is signified by a decrease in the first two behavioural measures and an increase in time spent on the bottom (Brown and Smith, 1997). Finally, all trout measurements were done live.

Data were analyzed using a MANOVA.

## **Results:**

### *Cichlids:*

Both treatment and donor group size had a significant effect on the change in anti-predator behaviour in juvenile convict cichlids ( $F_{3,163}=2.99$ ,  $p=0.033$ ;  $F_{5,165}=4.90$ ,  $p<0.001$  respectively). There was also a significant interaction between treatment and donor group size ( $F_{5,165}=2.82$ ,  $p=0.018$ ), which is why further ANOVA analyses were done.

ANOVA results found a significant effect of treatment for area use and time spent moving, an effect of donor group size for time spent moving and foraging rate, and no significant interaction terms (Table 1). In general, these changes correspond to an increase in anti-predator behaviour (or a decrease in all behavioural measures) in response to the disturbance cue at higher donor levels, with a few exceptions (Figure 1); at 12 and 15 donor levels, responses to the disturbance cue were often weaker than at 10 donors. As well, the subsequent repeated measures ANOVA done on undisturbed foraging rate data found a significant difference in pre and post stimulus values ( $F_{1,82}=13.52$ ,  $p<0.001$ ), but no interaction between the change in foraging rate and donor size ( $F_{5,82}=1.025$ ,  $p=0.41$ ). Fish receiving water from the undisturbed treatment also decreased foraging rate, indicative of an increase in anti-predatory behaviour (Figure 1).

### *Trout:*

In juvenile rainbow trout there was a significant effect of treatment on anti-predatory behaviour ( $F_{3,54}=12.34$ ,  $p<0.001$ ). However, unlike the cichlids, there was no effect of donor group size ( $F_{3,54}=0.93$ ,  $p=0.43$ ) or of the interaction between donor group size and treatment ( $F_{3,54}=1.99$ ,  $p=0.13$ ).

The addition of water from the disturbed tank corresponded with an increase in anti-predatory behaviour; I observed a decrease in time spent moving, reduced foraging rate, and an increase in time spent on the bottom as compared to undisturbed controls in both donor group sizes (Figure 2).

**Experiment 2: Do juvenile convict cichlids and juvenile rainbow trout show threat sensitive response patterns?**

*Cichlids:*

I repeated Experiment 1 using various 10.0 mL dilutions made from the disturbance cue of 10 donors: 100 % = 10.0 mL of disturbance cue; 75% = 7.5 mL of disturbance cue and 2.5 mL of distilled water; 50% = 5.0 mL disturbance cue, 5.0 mL water; 25% = 2.5 mL disturbance cue, 7.5 mL water; and 0% = 10.0 mL distilled water. The disturbance cue was collected in the same manner as mentioned in chapter one but no undisturbed control was used for this experiment. Sample sizes were 30 for all treatment groups except for the 100% group, which had a sample size of 29 due to poor visibility in one tank.

A MANOVA was followed by one-way ANOVA analyses. I also did a linear contrast since I had an *a priori* expectation that a graded response would exist.

*Trout:*

Trout were tested as described above for cichlids, using the same dilutions but with five trout donors instead of ten. I did this because preliminary results showed that trout responded overtly to five donors. The behavioural measures were the same as those used for trout in Experiment 1. Each treatment group had a sample size of 16 and the

statistics were done as mentioned in experiment two for cichlids.

## **Results:**

### *Cichlids:*

Overall there was a significant effect of concentration (percent disturbance cue) ( $F_{4,144}=6.09$ ,  $p<0.001$ ). Further ANOVA analyses also found percent disturbance cue to have a significant effect on area use, time spent moving and foraging rate behaviour (Table 2). However, linear contrasts were only significant for time spent moving and foraging rate, suggesting that the anti-predator response for area use is not graded. In fact, area use measures do not increase at higher disturbance cue concentrations as expected, whereas the results for both time spent moving and foraging rate do (Table 2; Figure 3).

### *Trout:*

As with cichlids, there was an overall effect of concentration ( $F_{4,75}=5.71$ ,  $p<0.001$ ). Subsequent ANOVA tests found concentration to be significantly different for time spent moving and foraging rate data but not for time spent on the bottom (Table 3). Likewise, linear contrasts were significant for time moving and foraging rate, suggesting a graded increase in anti-predator behaviour at rising disturbance cue concentrations for these two behavioural measures (Table 3; Figure 4).

## **Discussion:**

Both juvenile convict cichlids and juvenile rainbow trout appear to respond to a conspecific disturbance cue in a subtle and graded manner. Juvenile convict cichlids exhibited increased anti-predator behaviour in response to the disturbance cue as well as

to changes in donor group size. As predicted, smaller donor group sizes corresponded with a less intense response; a disturbance cue produced by fewer donors may not have been very strong and therefore may not have elicited overt changes in behaviour. However, the weakened behavioural response to 12 and 15 donors was unexpected; thus I must reconsider the assumption that larger donor numbers always correspond with greater concentrations of disturbance cue (Figure 1). One explanation for this may be that at such high population densities, disturbed donors were not 'distressed' because the level of risk to each individual was low. Brown et al. (2006) showed that in fish an individual's behaviour may change in such a way when group size is increased, although more work is needed to verify this.

Another unexpected finding was the behavioural response to undisturbed donors, particularly for foraging rate, that also increased in intensity at higher donor group sizes. This suggests that perhaps larger groups of undisturbed donors were releasing a disturbance cue and may help to explain why there was no significant effect of treatment for foraging rate. It is possible that undisturbed donors were stressed due to increased crowding conditions in their tanks. Since the disturbance cue is thought to be a metabolic waste product and possibly the same chemical released by stressed fish (Hazlett 1990a; Wisenden et al., 1995; Kiesecker et al., 1999; Jordao, 2004), it is plausible that crowded fish will release the cue in the absence of a predator. This could also explain why the response to larger disturbed donors (12 and 15) was less intense; if fish had been pre-stressed enough due to crowding, the cue may have been exhausted and largely removed from the tanks by the filter prior to testing (Hazlett, 1990a).

However, despite these discrepancies there was still an effect of treatment that suggests a disturbance cue exists, no matter the problems incurred by altering donor group size. This cue appears to evoke a subtle response in convict cichlids, which is unsurprising since previous work has come to similar conclusions (Jordao, 2004).

Trout responses to the disturbance cue were less complex than in cichlids. I found a significant effect of treatment but no effect of donor group size or of the interaction. This is because I only tested for a response at two donor levels, both of which produced a disturbance cue strong enough to elicit overt anti-predator responses. Why 5 donors can produce a response in trout but not in cichlids is unclear, although differences in natural habitat, receiver group size, diet, temperature, and species specific variability may be of importance.

In Experiment 2, I found an overall graded effect of the disturbance cue (Figure 3). Convict cichlids spent less time moving and reduced foraging as the concentration of disturbance cue increased. This relationship was linear, suggesting that the fish were behaving in a threat-sensitive manner, trading off typical fitness related behaviours (for example, foraging) in favor of a predator avoidance strategy. Although area use was significantly different at varying disturbance cue concentrations, anti-predator responses did not increase in intensity at higher concentrations (Figure 3). Again, this suggests the existence of a graded response since only some anti-predator behaviours changed in response to the cue (Brown et al., 2006).

When I repeated Experiment 2 using trout, I found similar results to those of cichlids, suggesting that the disturbance cue also evokes a graded response in rainbow trout. Similarly, the treatment did not have a significant effect on all behavioural

measures (time spent moving and foraging rate only) and a gradual increase in anti-predator behaviour was found at higher disturbance cue concentrations for time moving and foraging rate data (Figure 4).

The observed graded response suggests that receiver fish are making threat-sensitive trade-offs. At low disturbance concentrations, no overt changes in anti-predator behaviours were measured. As disturbance cue concentrations increase, the level of risk also increases and both cichlids and trout respond by decreasing foraging and time spent moving. However, the level of risk may not be high enough for other typical anti-predator behavioural changes to occur. Similar threat-sensitive behaviour has been observed in other studies when prey are exposed to varying levels of risk (Helfman, 1989; Lima and Dill, 1990; Foam et al., 2005; Brown et al., 2006).

Together, my data corroborates previous work on disturbance cues that suggest the response to the cue is present and subtle in juvenile convict cichlids and juvenile rainbow trout (Jordao, 2004; Lebedeva et al., 1999). I also found the response to be graded as opposed to an all-or-none reaction, which was unsurprising given the low-risk nature of the cue (Wisenden et al., 1995; Chivers and Smith, 1998; Bryer et al., 2001; Jordao, 2004).

Figure 1: Mean ( $\pm$ SE) changes in anti-predator behaviour (post-pre) in juvenile convict cichlids in response to disturbed (grey bars) and undisturbed (white bars) donors at varying group sizes. N = 15 for each treatment, except for 15 donors: disturbed N = 14, undisturbed N = 13.

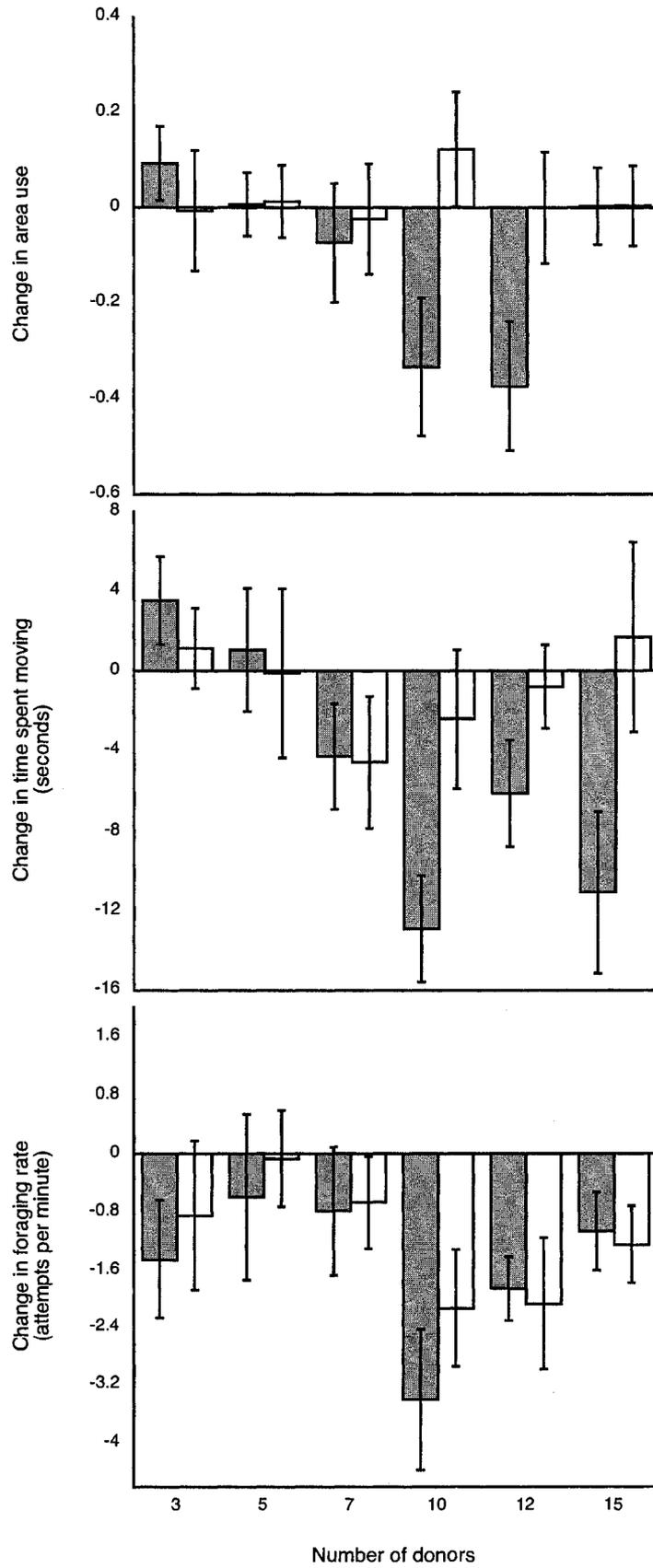


Figure 2: Mean ( $\pm$ SE) changes in anti-predator behaviour (post-pre) in juvenile rainbow trout in response to disturbed (grey bars) and undisturbed (white bars) donors at 5 and 10 group sizes. N = 15 for all treatment.

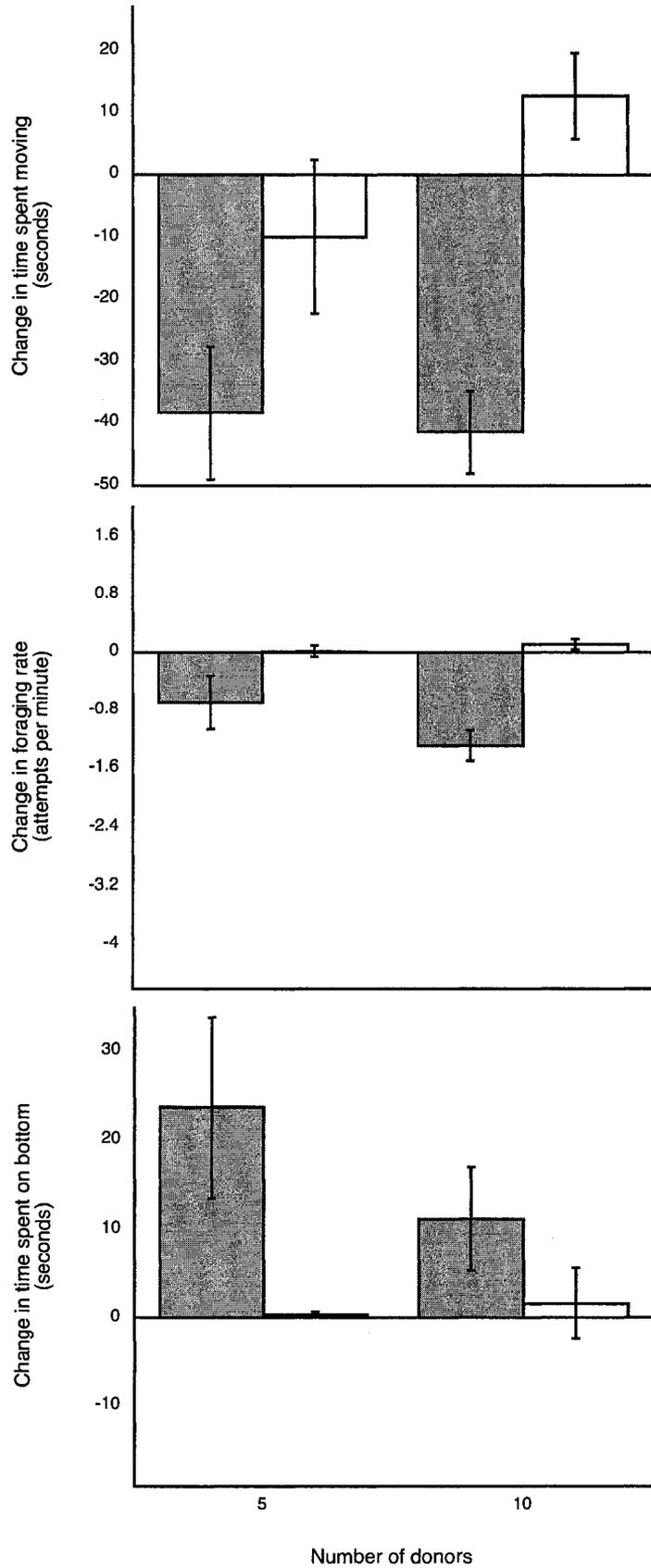


Figure 3: Mean ( $\pm$ SE) changes in anti-predator behaviour (post-pre) in juvenile convict cichlids in response to dilutions of disturbance cue. N = 30 for all treatments except the 100% treatment group, where N = 29.

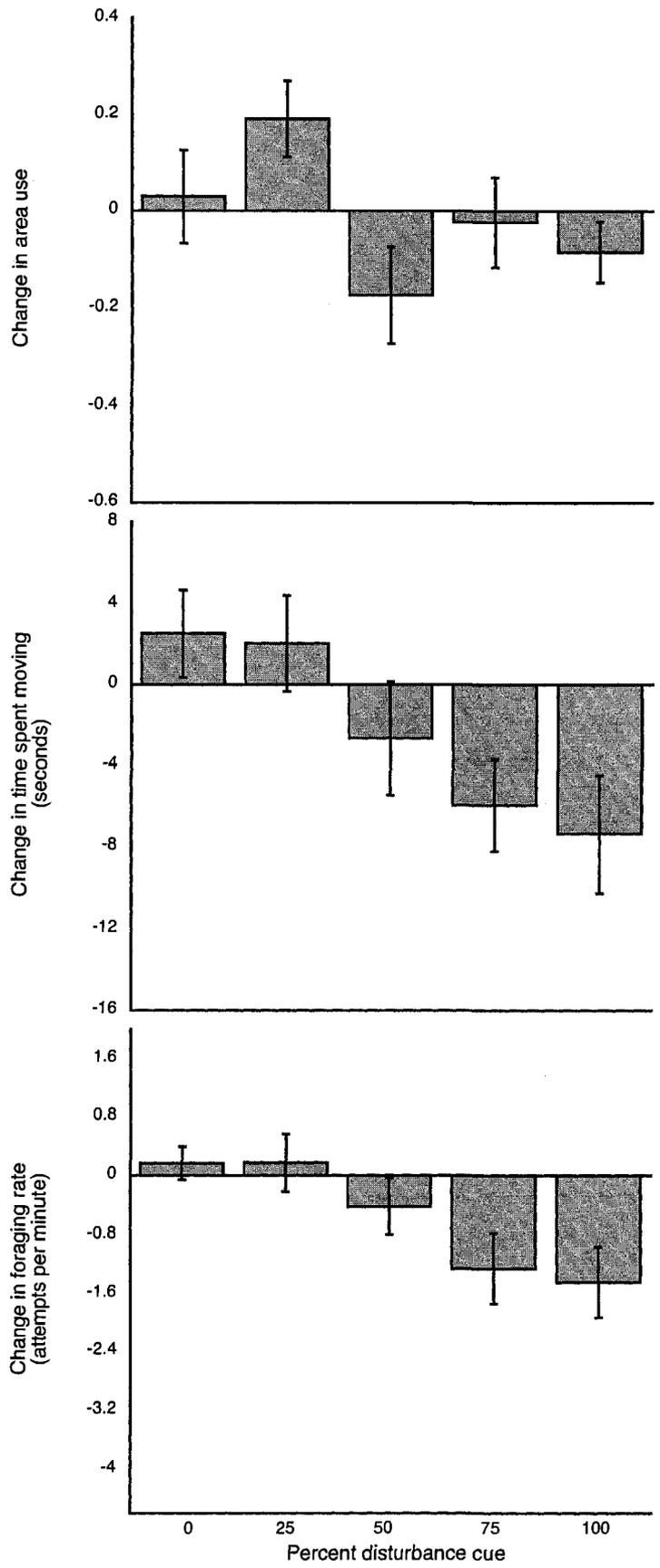


Figure 4: Mean ( $\pm$ SE) changes in anti-predator behaviour (post-pre) in juvenile rainbow trout in response to dilutions of disturbance cue. N = 16 for each treatment.

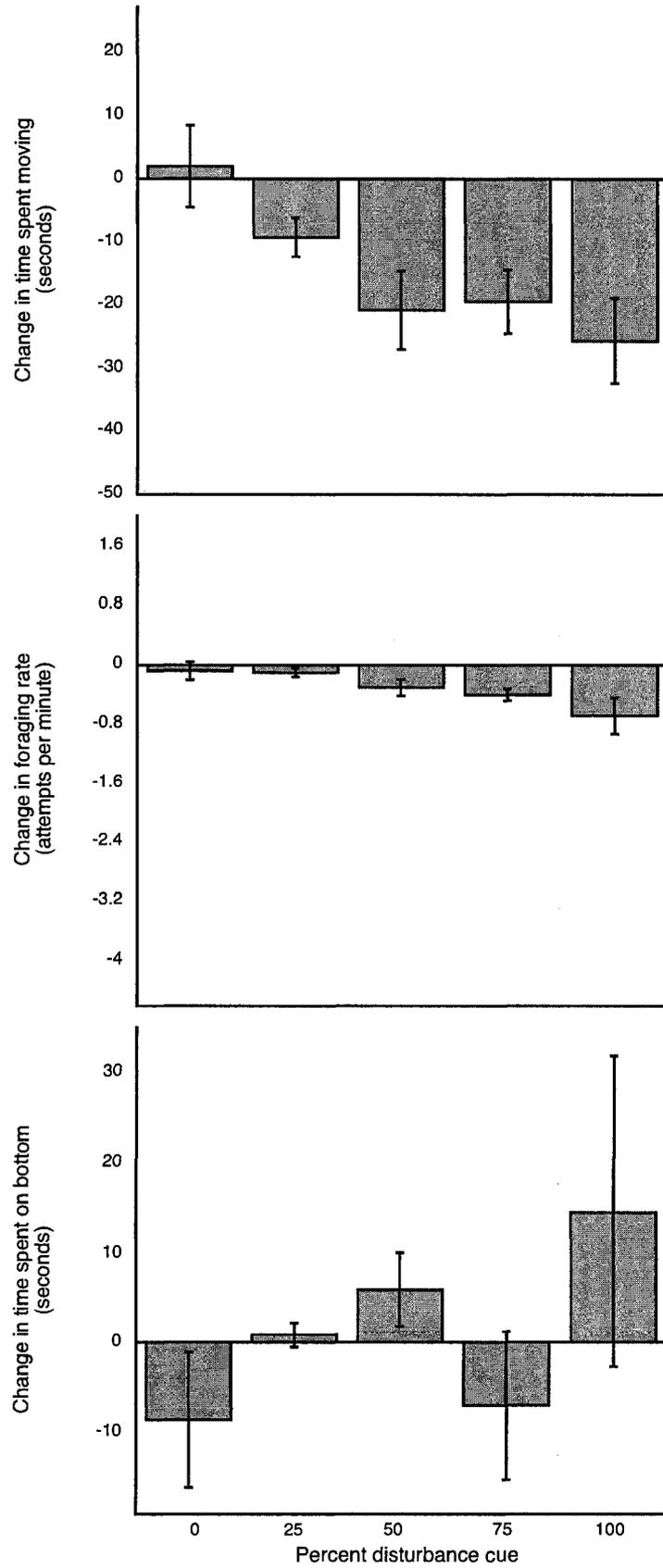


Table 1: ANOVA values for changes in anti-predatory behavioural measures when juvenile convict cichlids were exposed to various donor group sizes (3, 5, 7, 10, 12 and 15) of disturbed and undisturbed conspecifics.

<b>Area use</b>			
	F value	degrees of freedom (hypothesis, error)	p-value
Donor	1.23	5,165	0.30
Treatment	4.26	1,165	0.041
Donor*Treatment	2.15	5,165	0.062
<b>Time moving</b>			
	F value	degrees of freedom (hypothesis, error)	p-value
Donor	2.72	5,165	0.022
Treatment	5.07	1,165	0.026
Donor*Treatment	2.022	5,165	0.078
<b>Foraging rate</b>			
	F value	degrees of freedom (hypothesis, error)	p-value
Donor	2.37	5,165	0.042
Treatment	0.56	1,165	0.46
Donor*Treatment	0.24	5,165	0.95

Table 2: ANOVA and linear contrast values of changes in anti-predatory behavioural measures when juvenile convict cichlids were exposed to dilutions of disturbance cue (10 donors).

<b>Area use</b>			
	F value	degrees of freedom (hypothesis, error)	p-value
ANOVA	2.45	4,144	0.049
Linear term	2.56	1,144	0.11
<b>Time moving</b>			
	F value	degrees of freedom (hypothesis, error)	p-value
ANOVA	3.20	4,144	0.015
Linear term	13.27	1,144	< 0.001
<b>Foraging rate</b>			
	F value	degrees of freedom (hypothesis, error)	p-value
ANOVA	3.62	4,144	0.008
Linear term	12.12	1,144	0.001

Table 3: ANOVA and linear contrast values of changes in anti-predatory behavioural measures when juvenile rainbow trout were exposed to dilutions of disturbance cue (5 donors).

<b>Time moving</b>			
	F value	degrees of freedom (hypothesis, error)	p-value
ANOVA	3.83	4,75	0.007
Linear term	13.45	1,75	< 0.001
<b>Foraging rate</b>			
	F value	degrees of freedom (hypothesis, error)	p-value
ANOVA	3.17	4,75	0.018
Linear term	11.79	1,75	0.001
<b>Time on bottom</b>			
	F value	degrees of freedom (hypothesis, error)	p-value
ANOVA	1.04	4,75	0.40
Linear term	1.68	1,75	0.20

## **Chapter 3: Is the disturbance cue a metabolic waste product, such as ammonium?**

### **Introduction:**

A more complete understanding of the ecological importance of the disturbance cue is difficult without first understanding its chemical nature. Most studies on fish have suggested that the cue is likely ammonium excreted in the urine or across the gills, although no studies in fish have directly tested this (Wisenden et al., 1995; Jordao and Volpato, 2000; Bryer et al., 2001). These assumptions are based on previous findings in crayfish (*O. virilis*) and red-legged frogs (*R. aurora*) in which both species excreted ammonium after being disturbed and responded in an anti-predator manner when given ammonium directly (Hazlett, 1990a; Kiesecker et al., 1999).

About 80 to 90 percent of the metabolic waste produced by fishes is ammonia, with the remainder being urea (Wilkie, 2002). In freshwater fish, ammonia ( $\text{NH}_3$ ) is changed to ammonium ( $\text{NH}_4^+$ ) in the gill boundary and remains in that state in circum-neutral waters (Wilkie, 2002). Therefore, it is likely that the disturbance cue would be detected as ammonium. During an attack or in times of stress the metabolism of fishes may increase, thereby increasing metabolic waste (Kiesecker et al., 1999). In fact, it has been suggested the disturbance cue may be the same cue that is released in stressed fish (Wisenden et al., 1995; Chivers and Smith, 1998; Kiesecker et al., 1999; Jordao, 2004). To test the assumptions that the disturbance cue is a metabolic waste product (namely ammonium), I designed three experiments.

In my first experiment, I tested for the presence of ammonium in donor tanks before and after a visual disturbance using a colourimeter (Hach DR/890). A similar study using larval red-legged frogs found that tanks that were disturbed had approximately 0.1 mg/L higher levels of ammonium post disturbance compared to control tanks in which ammonium levels did not change (Kiesecker et al., 1999). Therefore, I predicted that by using a similar method of detection as Kiesecker et al. (1999), I would find comparable increases in ammonium concentration when fish were visually disturbed. I repeated this experiment in both convict cichlids and rainbow trout.

I followed the colourimetric tests by performing behavioural assays on cichlids and trout in response to concentrations of ammonium hydroxide similar to those reported by Kiesecker et al. (1999). If ammonium is a major component of the disturbance cue, I predicted an increase in the anti-predator response to higher ammonium concentrations compared to distilled water controls.

Finally, I tested to see if the cue was conserved across the two species. A variety of fishes such as ostariophysans, salmonids and cichlids respond to conspecific damage-released alarm cues but in the absence of learning they will not respond to cues from distantly related taxa (Wisenden and Sargent, 1997; Mirza and Chivers, 2001; Brown et al., 2003). However, if the disturbance cue is indeed a metabolic waste product in all fishes, I hypothesized that the same chemical would be recognizable by all fish species. This is true of Hazlett's (1990a) work, where it was found that crayfish respond to disturbance cues from the leech *Macrobdella decora*, the Iowa darter *Etheostoma exile*, and rock bass, *Ambloplites rupestris*, all of which excrete ammonia as their main metabolic waste product.

To test if the disturbance cue is recognizable from heterospecific donors, I gave juvenile rainbow trout disturbance cues from juvenile convict cichlids and visa versa to determine if their behaviour is similar to responses from conspecifics. I predicted an increase in anti-predator behaviour when either species was exposed to heterospecific disturbance cue. Such a result would suggest that the cue is potentially a metabolic waste product and that it is not species specific.

### **General Methods:**

Test fish were held under identical conditions as those reported in chapter two.

### **Experiment 3(a): detection of ammonium in disturbed and undisturbed tanks**

Test procedures were the same for convict cichlids and rainbow trout.

I held ten donor fish in each 20 L tank and collected water before and after disturbing half the tanks using a model predator (as per Chapter 2). Possible visual disturbance upon collection of the cue was unavoidable, and may be an extra source of disturbance in both disturbed and control tanks. However, preliminary tests led me to believe this would not confound my data.

I measured the ammonium concentration from all samples using a colourimeter (Hach DR/890) and ammonium test kits (Hach HCT 100 and Hach AmVer low range). Two types of test kits were used since the Hach HCT 100 was discontinued before testing was complete. However, both test kits used the salicylate method (as did Kiesecker et al., 1999) and the test ranges were similar for both: 0.05-1.5 mg/L for the HCT 100 and 0-2.5

mg/L for the AmVer kits. The sample size was ten for all treatment groups, however among undisturbed cichlids one highly negative outlier was removed from the analysis.

Student's t-tests were used to compare the average change in concentration (post-pre) of disturbed and undisturbed tanks. I did not attempt to compare cichlid and trout results directly since cichlids had a much higher biomass (see Chapter 2 General Methods). As well, I determined the power of detecting a change in 0.05 mg/L ammonium in the performed t-tests as described in Zar (1999).

## **Results:**

### *Cichlids:*

There was little change in ammonium concentration for both treatments: mean change  $\pm$  SE =  $0.0030 \pm 0.0083$  mg/L in disturbed and  $-0.0020 \pm 0.010$  mg/L in undisturbed tanks. The change was not significantly different between the two groups ( $t_{1,17}=0.46$ ,  $p=0.65$ ; Figure 5a).

### *Trout:*

As with the cichlid data, the change in ammonium concentration was minimal for both treatments: mean change  $\pm$  SE =  $-0.007 \pm 0.0047$  mg/L for disturbed and  $-0.010 \pm 0.0026$  mg/L for undisturbed. No significant difference in ammonium concentration was found between disturbed and undisturbed fish ( $t_{1,18}= 0.56$ ,  $p=0.58$ ; Figure 5b).

I found the probability of detecting a 0.05 mg/L change in ammonium to be at least 90 % in all cases tested.

### **Experiment 3(b): anti-predator behavioural response to ammonium**

#### **Collection of the cue:**

I obtained synthetic 14.53 M ammonium hydroxide, which breaks down into ammonium and water in an aqueous solution. The concentrated ammonium hydroxide was diluted using distilled water so that the final change in concentration of ammonium in the tanks would be approximately 0.01, 0.1 and 0.5 mg/L. These levels were chosen based on previous results with red-legged frogs (Kiesecker et al., 1999) that found an increase of approximately 0.1 mg/L ammonium post disturbance. New solutions were always made just prior to testing to minimize the breakdown of ammonium before it was administered to receiver fish.

#### **Experimental protocol:**

##### *Cichlids:*

The experimental protocol was the same as Experiment 1 except that I used various concentrations of synthetic ammonium (0.01, 0.1, 0.5 mg/L) and distilled water as my treatment groups. As well, I measured the initial ammonium concentration of all receiver tanks to test the baseline ammonium concentration. In order to avoid creating a disturbance while doing this, I used water taken up through the injection tube.

I performed a MANOVA followed by individual ANOVA comparisons. As well, I compared the control group to all others using a simple contrast test since I expected an increase in anti-predatory behaviour in response to ammonium.

##### *Trout:*

The methods and statistics were the same as mentioned above except that only 12 replicates were done. I used different behavioural measures than those mentioned for

trout in chapter two: average area use (body position on a three grid map that was drawn on the front of the tank), total time spent moving (seconds) and foraging rate (average value of attempts per minute). A decrease in all three behavioural measures signifies an increase in the anti-predatory response in rainbow trout (Brown and Smith, 1997).

### **Results:**

All receiver tanks had undetectable initial ammonium concentrations.

#### *Cichlids:*

I found a significant effect of treatment ( $F_{3,56}=2.88$ ,  $p=0.044$ ); however, further ANOVA analyses showed no significant differences between treatment groups for each behavioural measure (Figure 6; Table 4). As well, the simple contrast only found a marginally significant difference in foraging rate between the distilled water control and the 0.01 mg/L treatment (Table 5).

#### *Trout:*

There was a significant effect of treatment ( $F_{3,44}=4.08$ ,  $p=0.012$ ) and further ANOVA analyses found a significant difference in foraging rate ( $F_{3,44}=3.22$ ,  $p=0.032$ ) but not for area use or time spent moving (Table 6). However, the fish did not respond by increasing anti-predator behaviours at higher ammonium concentrations as was predicted; in fact, there is no pattern to the behavioural response for area use or foraging rate and the time spent moving results are opposite to what was expected (Figure 7). Simple contrasts only found a significant difference in time spent moving between the distilled water control and the 0.5 mg/L treatment, as well as in foraging rate between the control and the 0.01 mg/L treatment (Table 7).

#### **Experiment 4: cross-species response to the disturbance cue**

I measured the behavioural responses of cichlids and trout exposed to disturbed and undisturbed conspecific and heterospecific donors; the treatment groups (donor-receiver) were as follows: cichlid-cichlid, cichlid-trout, trout-cichlid and trout-trout. I used 10 donors for both cichlids and trout. All other methods are the same as reported in Experiment 1 in Chapter 2, with the exception of trout behavioural measures, which were the same as described in Experiment 3(b).

Data were analyzed separately for cichlid and trout receivers since the mean size and number of receivers was different for each. I did two 2-way MANOVAs using treatment (disturbed or undisturbed) and donor type as the factors.

#### **Results:**

In cichlids, there was no significant effect of treatment ( $F_{3,54} = 1.60$ ,  $p = 0.20$ ), donor type ( $F_{3,54} = 0.79$ ,  $p = 0.50$ ), or of the interaction between the two ( $F_{3,54} = 0.89$ ,  $p = 0.45$ ). However, in trout there was a significant effect of treatment ( $F_{3,54} = 6.85$ ,  $p = 0.001$ ), but not of donor type ( $F_{3,54} = 1.51$ ,  $p = 0.22$ ) or of the interaction ( $F_{3,54} = 1.37$ ,  $p = 0.26$ ). It appears that trout respond significantly to the disturbance cue from both cichlid and trout donors in an anti-predatory manner. Although not significant, cichlids also increased anti-predatory behaviour in response to conspecifics and somewhat to trout donors (Figure 8).

**Discussion:**

My results refute the hypothesis that ammonium acts as the major component of the disturbance cue, but suggests that the cue is potentially some type of metabolic waste product.

When donor fish were disturbed I found no significant change in ammonium concentration for both convict cichlids and rainbow trout (Figure 5), suggesting that ammonium is not released in significant amounts during times of stress. This is contrary to results in crayfish and red-legged frogs (Hazlett, 1990a; Kiesecker et al., 1999), despite using donors that had approximately six times greater biomass (g) per litre of water in my experiment (Chapter 2, General Methods; Kiesecker et al., 1999). Ammonium is the major metabolic waste product in fish (Wilkie, 2002) and it is therefore somewhat surprising that an increase in stress did not result in an increase in waste.

As mentioned in the methods, possible visual disturbance of the control tanks upon collection of the cue was unavoidable. However, since no significant change in ammonium concentration was detected, the collection of the cue did not seem to affect the results. Again, this could be because the collection of the cue did not disturb the fish or because they did not release excess ammonium upon disturbance.

Further evidence ammonium is not the major component of the disturbance cue in convict cichlids and rainbow trout is the lack of a consistent behavioural response to concentrations of synthetic ammonium, despite being given concentrations similar to those found to be excreted in red-legged frogs (Kiesecker et al., 1999). This lack of a consistent behavioural response (Figure 6 and 7) suggests that neither cichlids nor trout respond to ammonium in the same way that they respond to the disturbance cue (Figures

1, 2, 3 and 4). Although I cannot rule out that covert behaviour is occurring, this is unlikely when taken together with my previous experiments. In Experiments 1 and 2, I found that both cichlids and trout responded in an anti-predatory manner when exposed to the disturbance cue of 10 donors. However, 10 donors did not excrete ammonium in concentrations anywhere near to those given as treatment groups in Experiment 3(b) (Experiment 3(a); Figure 5).

Together, Experiments 3(a) and (b) suggest that ammonium is not a major component of the disturbance cue in convict cichlids and rainbow trout. However, the increased anti-predatory response in trout to heterospecific disturbance cue suggests that the cue, whatever it is, is not species specific, unlike the alarm cue (Wisenden and Sargent, 1997; Mirza and Chivers, 2001; Brown et al., 2003). Although cichlids did not respond strongly to heterospecific donors, this may be due to a concentration effect rather than an inability to detect trout disturbance cue, since no attempt to control for donor or receiver size was made between the two species. Thus, it is likely that cichlid donors, which were much larger than trout donors, were producing more cue. As seen in Experiment 2, a greater amount of cue should elicit stronger anti-predatory behaviour in accordance with the threat-sensitivity model (Helfman 1989). Nevertheless, the lack of specificity in trout concurs with previous suggestions that the disturbance cue is a metabolic waste product (Hazlett, 1990a) although future work is needed to verify the response in cichlids.

Future work should look into the possibility that other metabolic waste products, such as urea, are important components of the disturbance cue. Urea may be a good starting point since it is also released as a metabolic waste product in small amounts in

most fishes (Wilkie, 2002). According to Wilke (2002), some teleost fish excrete urea in distinct pulses under stressful conditions and its ecological relevance is unknown.

However, Lebedeva et al. (1999) suggests that 'chemical alarm substances' released in the urine or excrement of stressed rainbow trout were low molecular weight, thermostabile compounds. This suggests the cue is neither ammonium nor urea since neither is thermostabile (Ammonium Hydroxide MSDS, 2005; Urea MSDS, 2005). However, the manner in which the cue was collected makes it difficult to say if they were looking at a disturbance cue and not an alarm cue. Nonetheless, it may offer another starting point for future research.

Despite previous studies that suggest ammonium is a major component of the disturbance cue, it does not appear to be so in fish; ammonium was not excreted in concentrations similar to those found by Kiesecker et al. (1999) in disturbed frogs and no consistent anti-predatory behaviour was observed in response to synthetic ammonium. However, further understanding of the cue will require determining the major functional chemical involved. My results concur with previous work that suggest it is some type of metabolic waste product; therefore, future experiments should test for the presence of urea, a minor waste product, in disturbed fish.

Figure 5: Mean ( $\pm$ SE) ammonium concentration (mg/L) before (grey bars) and after (open bars) the disturbance of juvenile convict cichlids (a) and juvenile rainbow trout (b) compared to an undisturbed control when donor group size equaled ten; N = 9 in cichlids and 10 in trout.

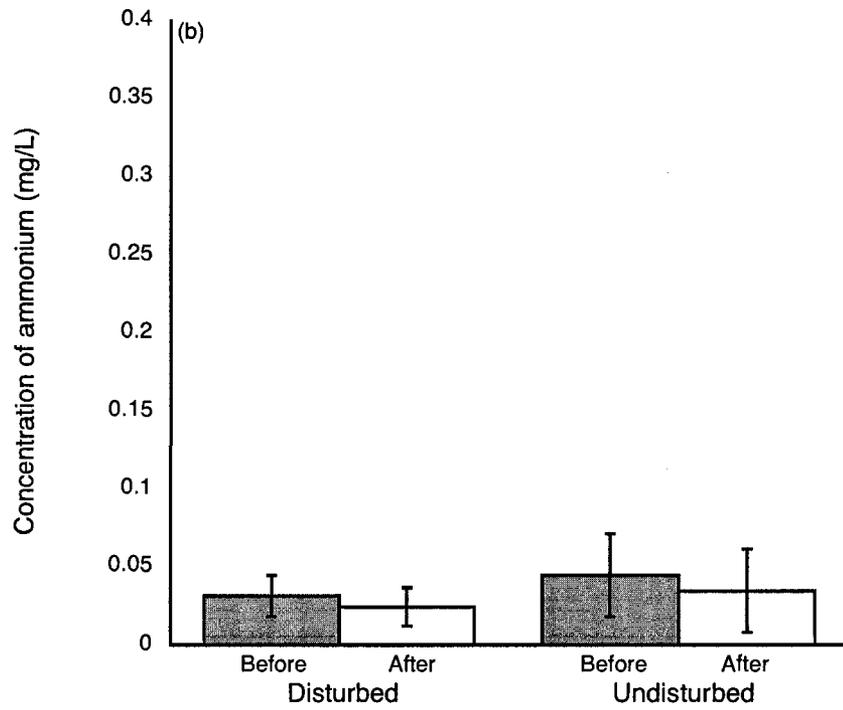
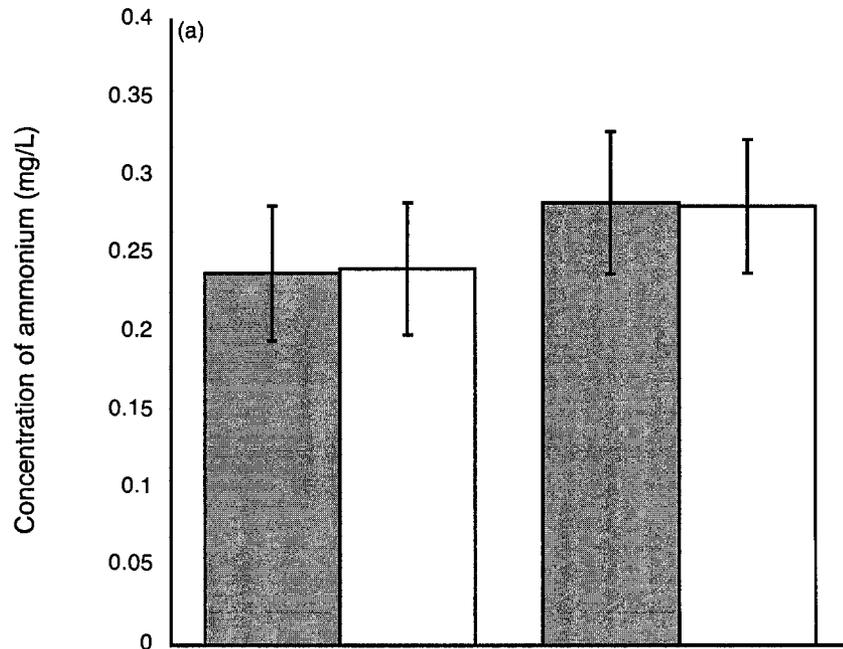


Figure 6: Mean ( $\pm$ SE) changes in anti-predator behaviour (post-pre) in juvenile convict cichlids in response to varying concentrations of ammonium. N = 15 for all treatments.

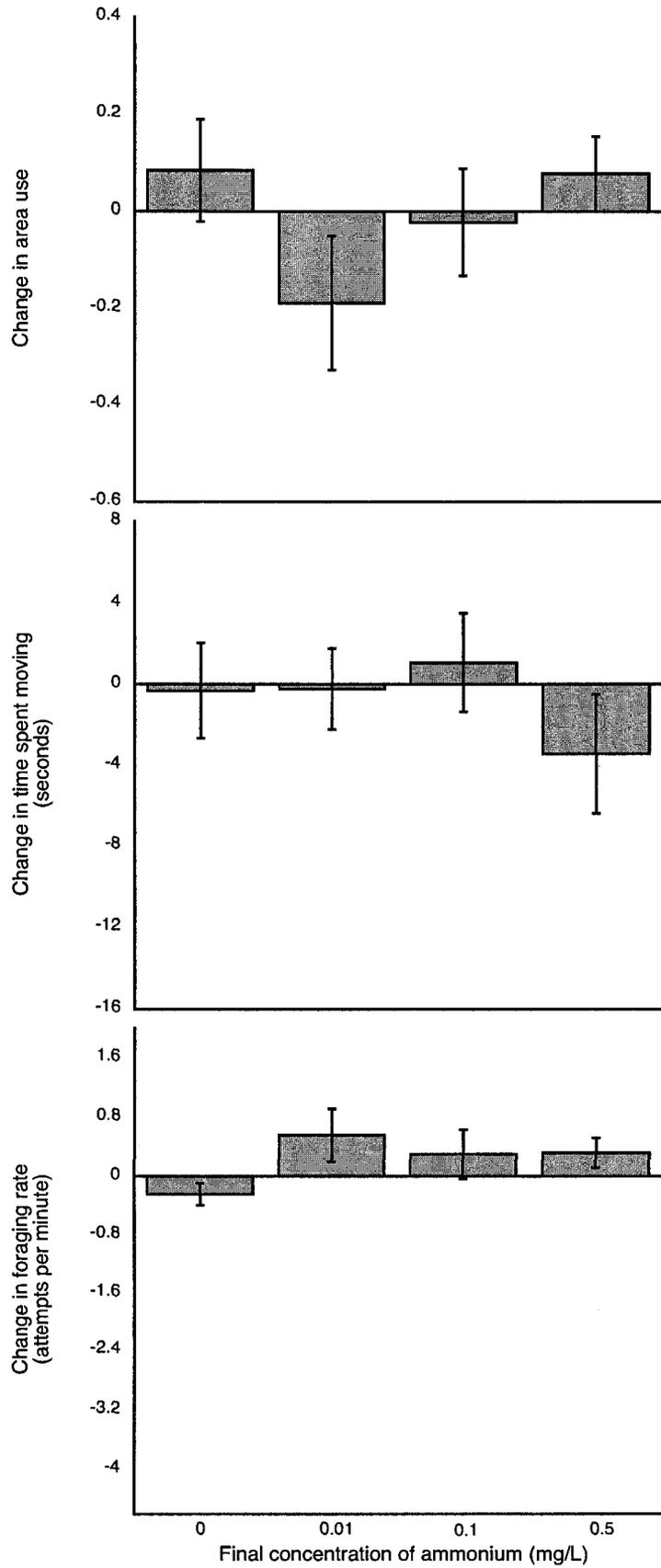


Figure 7: Mean ( $\pm$ SE) changes in anti-predator behaviour (post-pre) in juvenile rainbow trout in response to varying concentrations of ammonium. N = 12 per treatment.

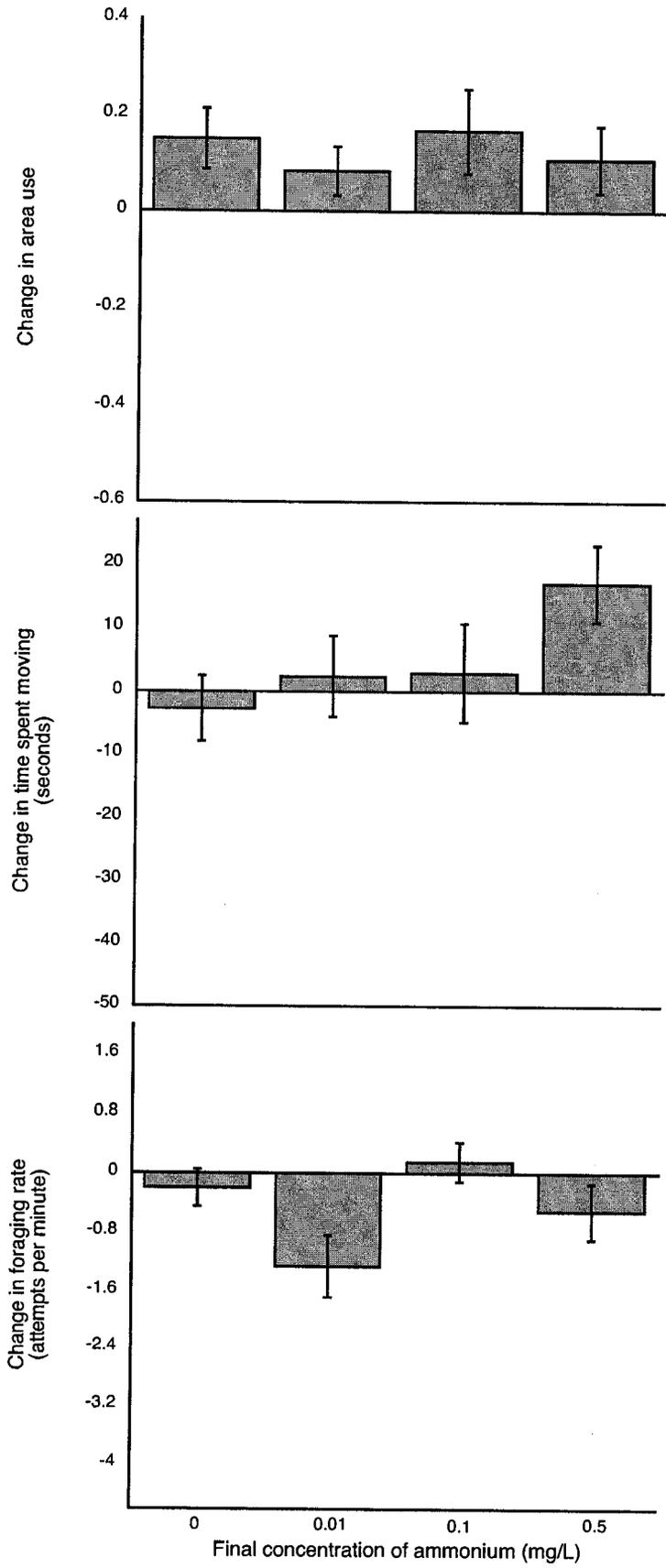


Figure 8: Mean ( $\pm$ SE) changes in anti-predator behaviour (post-pre) in juvenile convict cichlids and juvenile rainbow trout in response disturbed (grey bars) and undisturbed (white bars) conspecific and heterospecific donors. N = 15 per treatment.

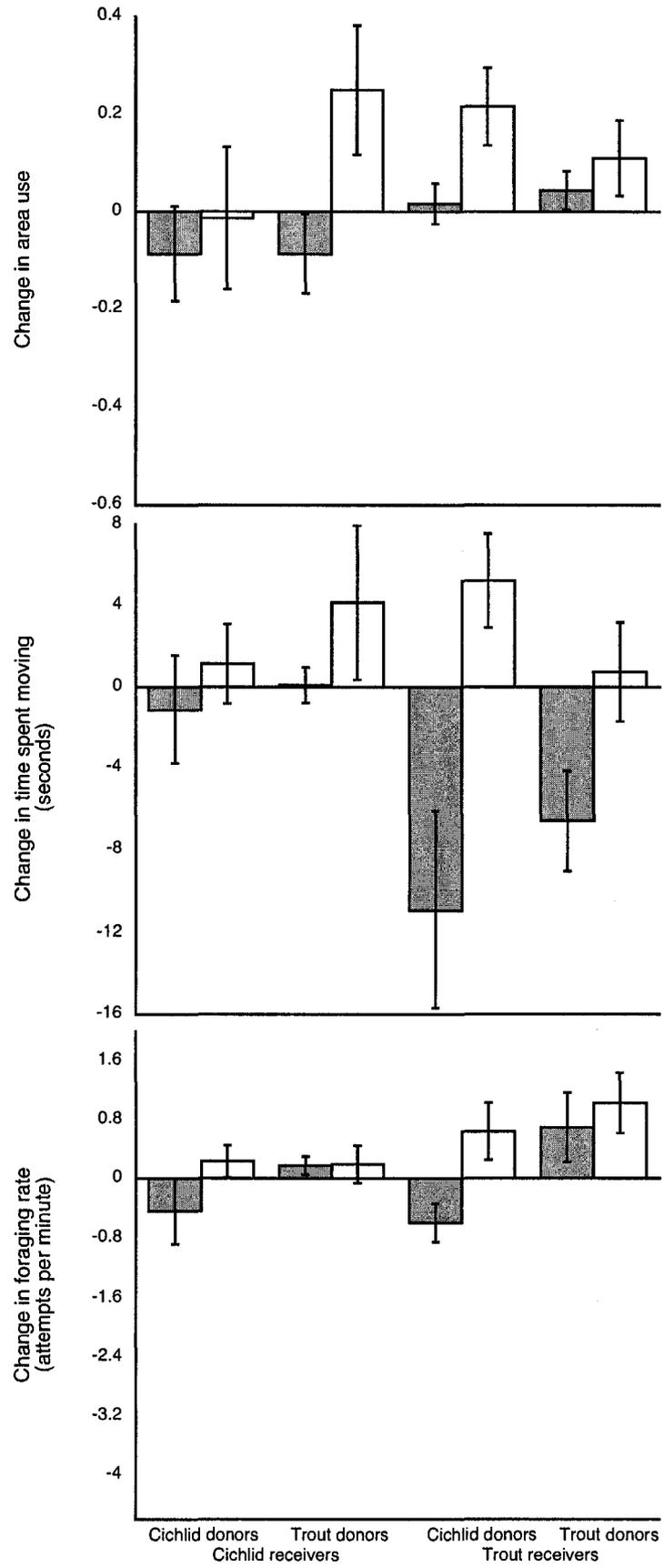


Table 4: ANOVA results for change in anti-predator behavioural measures when juvenile convict cichlids were exposed to dilutions of ammonium hydroxide.

<b>Area use</b>			
	F value	degrees of freedom (hypothesis, error)	p-value
ANOVA	1.35	3,56	0.27
<b>Time moving</b>			
	F value	degrees of freedom (hypothesis, error)	p-value
ANOVA	0.60	3,56	0.62
<b>Foraging rate</b>			
	F value	degrees of freedom (hypothesis, error)	p-value
ANOVA	1.48	3,56	0.23

Table 5: Simple contrast significance values when comparing the change in anti-predator behavioural measures of juvenile convict cichlids exposed to dilutions of ammonium hydroxide.

Contrast comparisons (NH <sub>4</sub> <sup>+</sup> mg/L)	Area use	Time moving	Foraging rate
0.5 vs 0	p=0.97	p=0.37	p=0.16
0.1 vs 0	p=0.49	p=0.69	p=0.18
0.01 vs 0	p=0.083	p=0.98	p=0.046

Table 6: ANOVA results for change in anti-predator behavioural measures when juvenile rainbow trout were exposed to dilutions of ammonium hydroxide.

<b>Area use</b>			
	F value	degrees of freedom (hypothesis, error)	p-value
ANOVA	0.30	3,44	0.82
<b>Time moving</b>			
	F value	degrees of freedom (hypothesis, error)	p-value
ANOVA	1.75	3,44	0.17
<b>Foraging rate</b>			
	F value	degrees of freedom (hypothesis, error)	p-value
ANOVA	3.22	3,44	0.032

Table 7: Simple contrast significance values when comparing the change in anti-predator behavioural measures of juvenile rainbow trout exposed to dilutions of ammonium hydroxide.

Contrast comparisons (NH <sub>4</sub> <sup>+</sup> mg/L)	Area use	Time moving	Foraging rate
0.5 vs 0	p=0.67	p=0.035	p=0.51
0.1 vs 0	p=0.86	p=0.54	p=0.47
0.01 vs 0	p=0.50	p=0.58	p=0.030

## **Chapter Four: Disturbance cues: “to infinity and beyond!”**

### **Discussion:**

Both juvenile convict cichlids and juvenile rainbow trout respond to disturbance cues in a subtle and graded manner. This is not surprising given the low-risk nature of the cue and support from previous studies (Wisenden et al., 1995; Chivers and Smith, 1998; Lebedeva et al., 1999; Bryer et al., 2001; Mirza and Chivers, 2002; Jordao, 2004). However, such studies have also suggested that ammonium is likely a major component of the disturbance cue (Hazlett, 1990a; Kiesecker et al., 1999), whereas my results do not support this. I found no significant change in ammonium in disturbed donor tanks and no consistent behavioural response to low levels of ammonium. Despite this, my results do suggest that the cue may be metabolic in nature since trout responded to a heterospecific disturbance cue (Hazlett, 1990a).

Future work should examine the ecological relevance of disturbance cues. How do they fit in to the big picture of predator avoidance? Under what mechanisms did they evolve? Are they an early warning system that may aid in learning and increased survival? Work is already being conducted in Grant Brown's lab to address some of these questions; in his 490 study, Bryan Fridman examined how disturbance cues may aid in learning. He found that juvenile rainbow trout conditioned with the disturbance cue plus alarm cue and predator odour later retained recognition of the predator odour alone. In fact, the anti-predator response was higher in fish conditioned with both the disturbance cue and the alarm cue than with those that were conditioned with only the alarm cue (unpublished data). As well, Chris Elvidge and Sabrina Lo are currently studying the role

of disturbance cues in the predation sequence by examining how juvenile rainbow trout respond to high and low concentrations of alarm cue when first given a disturbance cue stimulus.

However, to fully understand the disturbance cue, future work should determine what is the major functional chemical involved. Evidence supports the claim that the cue could be a metabolic waste product of some type (experiment four; Hazlett, 1990a), although more tests are needed to verify this. One such study could look at the effects of a high protein diet versus a low protein diet in donor fish. If the cue is a metabolic waste product, we would expect that donors with high protein diets would cause receivers to respond in a stronger anti-predatory manner. As well, future studies should examine urea and other secondary metabolic waste products as potential candidates for the disturbance cue.

Greater knowledge surrounding disturbance cues will increase comprehension of the complexity involved in chemical signaling and predator avoidance. It may aid in understanding fish responses in the wild, where multiple cues such as the disturbance cue, the alarm cue, and predator odours are present alone or in combination at varying concentrations. It may also further our knowledge on how fish learn and pass on important information (whether intentional or not) and what trade-offs are made. Since so little is known about disturbance cues to date, numerous possibilities exist for further research.

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