

**Behavioural and ecological implications of ambient acidification on the  
chemosensory alarm functions in juvenile salmonids**

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of  
Biology

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## Abstract

Behavioural and ecological implications of ambient acidification on the chemosensory alarm functions in juvenile salmonids.

Antoine Leduc  
Concordia University, 2007.

Alarm cues play important roles allowing prey individuals to act with context appropriate responses, increasing their chance to survive predators. One such type of alarm cue is chemical in nature and typically released following mechanical damage to the skin as would occur during a predation event. These damage-released chemical alarm cues are known to mediate local predation risks in many freshwater fish species. Under weakly acidic conditions (pH ~ 6.0) however, individuals exposed to chemical alarm cues have been shown to be impaired in their ability to detect these cues and respond with species-typical alarm behaviour. However, this effect has been demonstrated in only a single field study. In my first chapter, I conducted field observations in nursery streams ranging in pH from 5.71 to 7.49 on two year-classes (young-of-the-year and parr) of wild juvenile Atlantic salmon (*Salmo salar*). I assessed if the detection of these chemical alarm cues was dependent on the ambient pH or if variations in the detection occurred between populations regardless of the acidity levels. Salmon present in any acidified streams did not respond to alarm cues while those in neutral streams exhibited species-typical alarm responses. Secondly, I conducted experiments to further assess whether population or environmental differences was most likely to explain the loss of response to chemical alarm cues observed under acidic conditions. Thus, I conducted a reverse transplant experiment between salmon populations found under neutral (pH range ~ 7.0 – 7.3) and weakly acidic streams (pH

range ~ 5.8 – 6.3). I later assessed if five different populations of salmon produced chemical alarm cues enabling consistent antipredator behaviour in a receiving population. My results showed that population differences did not account for the observed difference in alarm response, where ambient acidity created a behavioural impediment to normal chemical alarm function. I then determined at which pH value the loss of alarm function occurs. Using juvenile rainbow trout (*Oncorhynchus mykiss*) in a laboratory study, my results showed that between 6.4 and 6.2 (pH unit), a steep decrease in alarm behaviour occurred despite the introduction of chemical alarm cues suggesting a graded loss of response with increasing acidity. In my fourth chapter, I wanted determine if the learning of a novel odour could occur when paired with chemical alarm cues, under both neutral and acidic conditions. I showed that although it was possible to condition salmon under neutral conditions, no conditioning occurred under acidic conditions. I finally determined under laboratory conditions if a survival cost (increased mortality) from predation exists for juvenile rainbow trout exposed to acidified or neutral alarm cues in the presence of a predatory largemouth bass (*Micropterus salmoides*). Trout exposed to acidified alarm cues had a significantly shorter survival time when compared to trout exposed to neutral alarm cues. Altogether, these results suggest that even subtle chemical changes in ambient acidity may interfere with the use of chemical alarm signal in otherwise pristine conditions.

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## Contribution of Authors

The chapters of this thesis were, to a large extent, prepared as manuscripts for submission to peer-reviewed journals for publication. Dr. Grant E. Brown contributed to the planning and methodology of the studies, data analysis and editing of all manuscripts. Ellie Roh contributed to the data collection of the field studies and editing for the manuscripts presented in Chapter 1, 2, and 4. Camille Macnaughton and Frederic Benz contributed to the data collection of Experiment 1 and the editing for the manuscript presented in chapter 2. Mark C. Harvey contributed to the data collection of the field study and the editing of the manuscript presented in Chapter 1. Fabien C. Lamaze and Lindsay McGraw contributed to the data collection of the laboratory study and the editing of the manuscript presented in Chapter 3. Finally, Cindy Breau has contributed to the data collection of the field study presented in chapter 4.

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Chapter 3 is in press as:

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## **General Introduction**

Failing to avoid predation may be quite unforgiving for prey as it greatly reduces future individual fitness (Lima and Dill 1990). Not surprisingly, over evolutionary time, predation has become a strong selective force implicated in the evolution of morphological, physiological, life history and behavioural adaptations (Sih 1987). Over a prey animal's lifetime, the risk of predation may vary greatly seasonally, daily or even on a minute by minute basis (Lima and Dill 1990). Thus, the importance for prey to be attuned to the prevalent risk is critical for its survival against predators. However, as animals must accomplish more in their lifetime than avoiding predators, they must balance the conflicting demands of predator avoidance against fitness enhancing activities such as feeding, mating and territorial defence. These conflicting demands may be resolved based on a prey's ability to reliably assess predation risk (Brown and Chivers 2005). For example, when an individual prey perceives a situation as 'risky', it should adjust its behaviour accordingly by increasing antipredator behaviour (e.g. decreasing its conspicuousness or avoiding the area of potential danger; Lima and Dill 1990, Smith 1999).

Risk assessment can be mediated by different sensory modalities that can be auditory, visual, mechanical, electrical or chemical in nature (Smith 1992, 1999). Moreover, individuals living in groups may benefit from signals sent by conspecific or heterospecific prey-guild members informing about the probability of a local danger (Lima and Dill 1990; Smith 1999). Typically, an alarm signal will be emitted when an individual detecting a danger warns others of such danger, even if the individuals receiving the signal are not directly threatened. Following this, informed individuals

should respond to the signal as they would to the danger itself (Chivers and Smith 1998; Smith 1999). For example, conspecific distress calls may warn squirrel monkeys (*Saimiri sciureus*) of a local danger, leading to increased motionlessness and vigilance (Griffin 2000). Importantly, the resulting responses should reduce the likelihood of being captured by predators.

In the aquatic environment, chemical alarm cues are especially useful to mediate local predation risks. This usefulness may be amplified in various environmental conditions such as high water turbidity, low light conditions and high structural complexity of the habitat (Wisenden 2000). As such, the importance of chemical alarm cues to mediate local risks in aquatic organisms has been extensively studied in the past decades in arthropods (Rochette et al. 1998; Wisenden and Millard 2001; Wisenden et al. 1999), amphibians (Woody and Mathis 1998) and fishes (reviewed in Brown and Chivers 2005). It is of interest that the benefits of chemical alarm cues in predation avoidance have been demonstrated in fish under laboratory and semi-natural conditions in which 'warned' individuals survived better than 'naïve' individuals (Mathis and Smith 1993; Mirza and Chivers 2000, 2003).

Given their critical role in the mediation of local predation risk, it is of concern that several fish species have shown impaired abilities to detect and/or respond to chemical alarm cues under sub-lethal acidic conditions (pH ~ 6.0; Brown et al. 2002; Leduc et al. 2004a). Other recent studies have demonstrated that under such a level of acidification, several components of fish behaviour, such as spawning migration (Ikuta et al. 2001), mating and nest digging behaviour (Kitamura and Ituka 2001), may be drastically repressed. These findings are of relevance for fish ecologists since such a



level of acidity was generally considered not to significantly affect the life histories and survival of freshwater fish, particularly salmonids (Lacroix et al. 1985; Gunn and Noakes 1986; Peterson et al. 1989). Of interest, Kitamura and Ikuta (2000) reported that the lethal concentration for 50% mortality in several juvenile salmonid to range from 3.7 to 4.1 (pH units) for 24h.

Thus, the goal of this thesis is to investigate the behavioural and ecological consequences of weak levels of acidification (pH ~ 6.0) on the alarm response of freshwater fish to chemical alarm cues using juvenile stream-dwelling salmonids (Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*; Salmonidae)) as model prey species. Juvenile stream-dwelling salmonids possess a suite of distinctive advantages for studying the response to chemical alarm cues under natural and laboratory conditions. First, studies have demonstrated that several salmonid species respond with species-typical alarm behaviour to conspecific chemical alarm cues (Brown and Smith 1997; Mirza and Chivers 2001a). Second, under natural conditions, juvenile salmonids (including Atlantic salmon) are typically 'sit and wait' foragers, guarding a feeding territory of relatively small area (Grant et al. 1998; Steingrímsson and Grant 2003), making it feasible to study and directly quantify their behaviour under natural conditions (Leduc et al. 2004a).

A single study has demonstrated the effects of weak acidification on the alarm response of juvenile brook trout (*Salvelinus fontinalis*) to chemical alarm cues under natural conditions (Leduc et al. 2004a). In this study, one population of brook trout was studied under neutral conditions versus one population under acidic conditions. While Leduc et al. (2004a) showed that trout did not respond to the alarm cues under acidic

conditions, differences in acid tolerance can exist between different populations/strains of salmonids (Robinson et al. 1976; Rosseland et al. 2001). For instance, some strains may be adequately suited to live in acidic conditions while others may suffer increased physiological stress leading to sub-optimal behaviour (Åtland 1998). Thus, the observed difference in alarm response between the two studied populations may be the result of differences in overall physiological condition and/or olfactory sensitivity rather than from a direct loss in chemical alarm function. In chapter 1, my goal was to assess whether different populations of juvenile Atlantic salmon found in weakly acidic streams were all similarly (i.e., with the same response intensity) impaired in their ability to detect and respond to chemical alarm cues. I predicted that if a loss of response to chemical alarm cues was attributable to a chemosensory impairment, all studied salmon populations under acidic conditions should fail to respond to the alarm cues (i.e., no significant difference in alarm response intensity between populations).

The ability to detect chemical alarm cues may vary across fish populations. For instance, differences in olfactory and gustatory sensitivity between fish populations occur, leading to inconsistent abilities to detect water-borne cues (Hara 1999). Therefore, the ability to detect and respond to chemical alarm cues may be affected by intrinsic sensory sensitivity differences rather than resulting from environmental acidity.

Likewise, the quantity and/or quality of the chemical alarm cues produced by prey fishes may be affected by individual body state. For example, compared to poorly fed alarm cue donors (i.e., the sender of the chemical alarm cues), well-fed donors produced chemical alarm cues eliciting a significantly greater intensity of antipredator behaviour in receiving individuals (Brown et al. 2004). In chapter 2, I assessed the contribution of

environmental acidity versus possible differences in the ability of different populations to detect or produce the chemical alarm cues. I conducted a reverse transplant experiment between neutral and acidic streams in which juvenile salmon were tested in their 'home' stream or introduced into a stream of different acidity. I also assessed whether chemical alarm cues of salmon populations inhabiting streams of two different pHs would elicit consistent alarm responses in a receiving population found under neutral conditions (the conditions in which the alarm response was previously measured). I predicted that if the chemosensory alarm function was impaired by weak acidification, all salmon (regardless of their origin) tested under acidic conditions should fail to respond to the alarm cues while under neutral conditions, salmon should respond to the alarm cues. Likewise, if the observed impairment is not linked to population differences in the production of the alarm cues, the chemical alarm cues from all salmon populations should elicit observable alarm responses in receiving individuals (providing that overall, individuals of a given population have similar detection abilities).

Significant fluctuations in ambient acidity occur under natural conditions following natural and anthropogenic acidic inputs (Komai et al. 2002; Baker et al 2004). For instance, seasonal changes in pH typically occur during the spring snowmelt during which ambient acidity may increase drastically (for example, 50-80% of the acidity is released in the first 30% of the snowmelt; Gunn 1986). Moreover, changes from circum-neutrality to acidity may occur following a single rain event, lowering the ambient acidity by almost single pH unit (Komai et al. 2002; A.O.H.C. Leduc, Concordia University, unpublished data). As such, prey individuals may be living in conditions of fluctuating environmental acidity. In chapter 3, my goal was to assess if an acidity threshold exists

for the loss of alarm response to chemical alarm cues. I also tested if such a loss of response occurred gradually as the acidity increased or if it occurred in an ‘all or nothing’ fashion.

Although it may seem counterintuitive for antipredator responses to be dependant upon experience, the ability to learn about previously unfamiliar predators has been demonstrated in a wide range of taxa including birds, mammals and fish (reviewed in Griffin et al. 2000; Brown 2003). The alarm response to chemical cues, however, does not require specific learning but can serve as reinforcement for the learning of other characteristics of predators, such as its odour (Smith 1992; Chivers and Smith 1997). Leduc et al. (2007a) have shown that under natural conditions, juvenile Atlantic salmon could learn to recognize a novel odour after it was paired with conspecific chemical alarm cues. In chapter 4, my goal was to investigate whether such acquired conditioning between chemical alarm cues and a novel odour can occur under acidic conditions. I predicted that if acidic conditions impair the detection of the alarm cues, no such conditioning would occur.

The ability to respond to chemical alarm cues may translate into greater survival for prey individuals (Mirza and Chivers 2000, 2001b, 2003). Consequently, it is suspected that any impairment in the ability to detect an alarm signal (including chemical alarm cues) should lead to an increased fitness cost. However, the inability to detect an alarm response may not necessarily imply that individuals are not gaining any information about the prevalent danger. Often, the intensity of an animal’s antipredator response should reflect the level of risk posed by the predator (Helfman 1989). When it comes to chemical cues, the concentration of alarm cues that an animal detects may be

used to mediate the intensity of its antipredator response. Thus, the minimum response threshold hypothesis predicts that under a certain level of perceived risk (i.e., concentration of chemical alarm cues), no observable response should occur (Brown et al. 2001a). Using chemical alarm cues, Mirza and Chivers (2003) have shown increased survival of prey exposed to concentrations of alarm cues that failed to trigger an observable alarm response. In chapter 5, my goal was to assess a direct ecological impacts of predation during the observed chemosensory impairment under acidic conditions. The result of this test would likely reveal insights of the possible degradation mechanism of the alarm cues. I conducted a staged-encounter experiment between rainbow trout (as prey) and largemouth bass (*Micropterus salmoides*; as predator). I predicted that if the alarm cues are rendered non-functional under acidic conditions, prey should suffer increased predation costs. Alternatively, if the chemical alarm cues are only partially degraded, prey may still gain survival benefits from the information provided by the alarm cues.

## **Chapter 1: Impaired detection of chemical alarm cues by juvenile wild Atlantic salmon (*Salmo salar*) in a weakly acidic environment. \***

### **Introduction**

Acidification of freshwater ecosystems has significant impacts on aquatic communities worldwide (Schindler 1988; Guerold et al. 2000). While in the past two decades anthropogenic acidic deposition has decreased by 30 - 40% in many industrialized countries (Stoddard et al. 1999), weakly acidic water bodies (pH ~ 5.5 - 6.0) are still widespread (Mallory et al. 1998; Jeffries et al. 2000; Doka et al. 2003). In fishes, acidification is known to induce behavioural changes such as increased area avoidance (Johnson and Webster 1977; Åtland 1998), inhibition of migration (Ikuta et al. 2001), inhibition of spawning (Kitamura and Ikuta 2001), reduced feeding (Lacroix et al. 1985) and impairment of chemosensory risk assessment (Brown et al. 2002; Leduc et al. 2004a, b). Several of these behavioural changes may represent sub-lethal effects of anthropogenic acidification. Despite its frequent occurrence, most studies investigating the effects of acidification on fish behaviour have concentrated on intermediate to severe acidification (pH values between 4.0 - 5.5; Lacroix et al. 1985; Gunn and Noakes 1986; Peterson et al. 1988) while the effects of weak acidification (pH of 6.0) has received much less attention (but see Ikuta et al. 2001).

A wide variety of aquatic vertebrates rely on chemosensory information to assess and avoid local predation risks (Smith 1992, 1999). Such risk assessment is mediated through the release and detection of chemical cues in the water column (Chivers and

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\* Leduc, A.O.H.C., Harvey, M.C., Roh, E., & Brown, G.E. (2006). *Canadian Journal of Fisheries and Aquatic Sciences*, **63**, 2356-2363.

Smith 1998; Wisenden 2000). One extensively studied group of such chemicals is the damage-released alarm cues (reviewed in Chivers and Smith 1998; Brown 2003). These cues are present in the epidermis of taxonomically diverse prey fishes and typically enter the water column following mechanical damage to the skin, as would likely occur during a predation event (Chivers et al. 1996; Brown and Smith 1997). Alarm cues have been studied in several species of salmonids including brook charr (*Salvelinus fontinalis*; Mirza and Chivers 2000, 2001a), brown trout (*Salmo trutta*; Mirza and Chivers 2001a), rainbow trout (*Oncorhynchus mykiss*; Mirza and Chivers 2001a; Leduc et al. 2004a) and chinook salmon (*Oncorhynchus tshawytscha*; Berejikian et al. 1999). Their detection by nearby conspecifics and some sympatric heterospecific prey-guild members may elicit a suite of short-term antipredator behavioural responses including decreased activity level, increased use of shelter and increased area avoidance (Smith 1992, 1999). Prey individuals responding to alarm cues have been shown to gain increased survival during staged-encounters with live predators (Mirza and Chivers 2000, 2001b, 2003).

The ability to detect and respond to these critically important chemosensory cues may be impaired under acidic conditions. For example, Brown et al. (2002) demonstrated in a laboratory experiment that a weak level of acidification (pH of 6.0) renders two Ostariophysan species, fathead minnow (*Pimephales promelas*) and finescale dace (*Phoxinus neogaeus*), unable to detect and respond to conspecific alarm cues. A similar loss of function of chemical alarm cues has been demonstrated for juvenile pumpkinseed sunfish (*Lepomis gibbosus*; Leduc et al. 2003) and rainbow trout (Leduc et al. 2004a). In all cases, the loss of response does not appear to be due to physiological damage to cue receptors, but rather to changes in the alarm cue molecule itself (Brown et

al. 2002).

Laboratory conditions, however, lack the ecological relevance of the challenges prey individuals face in their natural habitat and as such, controversy exists regarding the function of chemical alarm cues under natural conditions (see Magurran et al. 1996; Smith 1997). While several recent studies have shown that various prey fishes do rely on chemical alarm cues to assess risk under natural conditions (Brown et al. 1997; Wisenden et al. 2004), only a single field investigation has examined the potential effects of stream water acidification on the use of these cues. Leduc et al. (2004a) have shown that in a stream of a mean pH of 6.88, conspecific alarm cue injections elicited a significant increase in antipredator response in brook charr compared to a control stimulus. However, in a stream of a mean pH of 6.11, no change in response was observed following injections of the chemical alarm cues. Population and strain-dependant variations in acid tolerance exist in many fish species including salmonids (Robinson et al. 1976; Rosseland et al. 2001). While some strains may be adequately suited to live in more acidic conditions, others may suffer increased physiological stress leading to sub-optimal behaviour (Åtland 1998). Thus, studying a single population at each acidity level cannot exclude the possibility that differences in alarm response may be the result of population differences in overall physiological condition and/or olfactory sensitivity rather than from a direct loss in chemical alarm function. Therefore, the general ecological relevance of the effects of weak acidification on chemosensory assessment of damage-released alarm cues remains difficult to assess.

In this study, I assess if the alarm response to conspecific chemical cues of juvenile wild Atlantic salmon (*Salmo salar*) is correlated with the ambient pH in nursery



streams not directly disturbed by human activities (with the exception of potential acid precipitations). I test for the presence of an alarm response in young-of-the-year (YOY; 0+) and parr (1+) following the exposure to chemical alarm cues in streams of different pH. If a loss of function of chemical alarm cues is linked to alterations in the alarm cue itself rather than population differences in acid tolerance, I predict that prey fish present in any of the acidic streams should show a reduced alarm response compared to fish present in neutral streams. Likewise, I would expect no difference in overall response intensity between populations present in streams of a similar acidity level.

I chose juvenile Atlantic salmon to investigate these questions owing to its territorial and site fidelity behaviour (Grant et al. 1998; Steingrímsson and Grant 2003) allowing for visual observations to be conducted with ease in its habitat. I compared four typical behavioural changes associated with antipredation responses (see below) for two year-classes, YOY and parr, in six sites of five different streams ranging in acidity from pH 5.74 to 7.33.

## **Material and methods**

### ***Test sites***

This experiment was conducted in Northumberland County, New Brunswick, Canada in five different streams (Catamaran Brook, Little Southwest Miramichi River, Otter Brook, Devil's Brook and Correy Creek; Table 1.1) between June 14<sup>th</sup>– 29<sup>th</sup> and August 7<sup>th</sup>– 23<sup>rd</sup> 2003. In these streams, a site of approximately 50 m in length (Figure 1.1) was chosen and its physical characteristics (canopy cover, distance from the riverbank and substrate type; Table 1.2) were recorded (see below). Because Correy Creek is a tributary of

Devil's Brook, I chose to subdivide Devil's Brook into two test sites: one above and below the mouth of Correy Creek. These two sites on Devil's Brook (named Devil's Brook 'High' and Devil's Brook 'Low', respectively) had two distinct mean pHs (one-way analysis of variance,  $p < 0.05$ ) but were otherwise similar in their physical characteristics (Table 1.2).

After each trial, the pH and water temperature were recorded (using a portable Accumet® EW-59333-20 pH meter) as well as the surface current speed (using a Flo-Mate velocity meter; Marsh McBirney Inc.), the depth (using a one meter ruler), the cloud cover and the canopy cover. The cloud cover was estimated by the same observer throughout the entire experiment by assessing the percentage of sky that was covered by clouds. The canopy cover was determined by estimating the proportion of sky that was blocked by branches and trees directly above the point of stimulus injection in a two metres radius. Overall, test sites were grouped in two distinct categories, neutral or acidic, as a function of their mean pH (Table 1.2). Catamaran Brook, Little Southwest Miramichi River and Otter Brook were considered neutral (pH range of 6.96 – 7.33) while Devil's Brook 'High', Devil's Brook 'Low' and Correy Creek were considered acidic (pH range of 5.74 – 6.09). With the exception of the pH, no significant difference between the measured characteristics of the two groups of test sites was found (Table 1.3). Also, there was no significant difference in mean pH in each respective group of streams (neutral or acidic) between June and August (Table 1.3).

### ***Stimulus collection***

Skin from juvenile Atlantic salmon was collected as a source of the alarm cue substance on June 13<sup>th</sup> and August 5<sup>th</sup> 2003 from Little Southwest Miramichi River. Less than an hour after their capture, these Atlantic salmon were killed with a single blow to the head (in accordance to Concordia Animal Care Committee Protocol AC-2002-BROW) and skin fillets from both sides of their body were removed and immediately placed into an ice-chilled container filled with stream water of pH 7.04 and 7.08 (June and August, respectively). In June, I collected 327.74 cm<sup>2</sup> and 332.21 cm<sup>2</sup> of skin (parr and YOY, respectively) while in August I collected 348.21 cm<sup>2</sup> and 319.80 cm<sup>2</sup> of skin (parr and YOY, respectively). Fillets were homogenized and diluted with stream water producing alarm cue solutions ranging from 0.150 - 0.158 cm<sup>2</sup>•mL<sup>-1</sup> (Table 1.4).

This procedure has been shown to elicit a consistent antipredator response in cyprinids (Brown et al. 2002), centrarchids (Leduc et al. 2003) and in salmonids (Brown and Smith 1997, 1998; Mirza and Chivers 2002). The resulting solution was frozen in 25 mL aliquots at -20 °C until needed. As a control, stream water was also frozen in 25 mL aliquots.

### ***Experimental protocol***

Field observations were conducted between June 15<sup>th</sup> – 29<sup>th</sup> and August 7<sup>th</sup> – 29<sup>th</sup> 2003 using a method used by Leduc et al. (2004a) (modified from Steingrímsson and Grant 2003). Observation trials were conducted on focal test fish (found while snorkelling on the test sites) from approximately 1.5 m upstream of the test fish, at an angle of 45 degrees relative to the water current. This positioning was used to minimize visual

obstruction of the focal fish and to reduce interference from drifting particulate matter. To ensure that the fish was behaving ‘normally’ (i.e., it had resumed feeding and moving), it was left to acclimate to the observer’s presence for a minimum of two minutes before any observations were recorded. Trials were 20 minutes in length and divided in two 10-minute pre- and post-stimulus injection periods. After the first 10-minute elapsed (pre-stimulus), the observer injected the stimulus (either stream water or alarm cue solution for the control and the experimental treatments respectively) using a 60 mL syringe at a constant rate of  $6 \text{ mL}\cdot\text{s}^{-1}$ . The stimulus was injected from approximately 1 – 1.5 m upstream of the test fish. The experimental treatment order and the year-class subjects were completely randomized using a coin flip while the overall order of the test sites surveyed was partially randomized. All trials were conducted and videotaped by the same observer using an underwater “Sea View™” camera. As the camera was positioned between 1 to 1.5 m from the focal test fish, small-scale behavioural responses could easily be monitored. In total, 144 trials were conducted (six replicates per treatment in six sites with two year classes). Each trial was conducted on a single fish that was used only once. To ensure that the injected stimulus reached the test fish, dye injection tests (commercial 2% milk) were conducted. These tests were done after the completion of 16 randomly assigned experimental trials in which the same experimental protocol was used. For all dye injection trials, the injected dye reached the test fish suggesting that our injected stimuli were also reaching the test fish. Smith (1999) reported that a single exposure to alarm cue was sufficient to elicit an alarm response.

### ***Behavioural measures***

To assess the intensity of an alarm response from exposure to a stimulus as well as to increase the power of detection, five behavioural modalities were quantified: the number of feeding attempts, the number of aggressive interactions, the total time spent in motion (in seconds), the total time spent motionless on the substrate (in seconds) and the total time spent absent (in seconds). These behavioural responses were quantified by viewing videotapes on a 14-inch (35 cm) Toshiba™ flat screen monitor. All trials were viewed twice by the same observer. I chose these above-mentioned behavioural measures as they allow to quantify the intensity of an alarm response in several fish species (Mirza and Chivers 2002; Leduc et al. 2004a; Wisenden et al. 2004) and may confer survival benefits to individuals during predator encounters (Mirza and Chivers 2003). A feeding attempt was defined as a movement of at least half a body length toward a drifting particle or a particle on the substrate, where a biting attempt occurred. An aggressive interaction was defined as a movement of at least a body length toward another fish with or without a biting attempt. Time in motion was measured when an individual changed its location by at least a body length. The time on substrate was measured when an individual was laying on the substrate without changing its location. Finally, the time absent was defined as the time when a fish was no longer observable on its stimulus injection location (either away or hiding). For the latter parameter, if the test fish was seen more than three meters away from its testing location, it was considered absent. This cut-off distance was used since the observed displacements of territory holding juvenile Atlantic salmon are generally under this distance (Steingrímsson and Grant 2003). In numerous cases, it was not possible to differentiate between hiding and fleeing therefore these two

responses were grouped together. Because I cannot assess whether certain response variables convey a stronger indication of fright, I interpreted the appearance of any of these behavioural patterns as an alarm response.

### ***Statistical analysis***

The results were obtained by subtracting the pre- values from the post- values, thus giving the difference between the measured behavioural parameters (see above). I tested for any overall effect of pH with a nested multivariate analysis of variance (MANOVA) using treatment (stream water versus alarm cue) and pH (neutral versus acidic) as independent variables. I nested the variable 'stream' in their respective pH level (neutral or acidic). Using subsequent analysis of variance (ANOVA), the effects of pH and stimuli were further investigated. The behavioural response of individuals was directly compared between control and experimental stimuli under neutral and acidic conditions for each year class (parr and YOY). I used SPSS 11 to perform all statistical analysis.

### **Results**

For both parr and YOY, a significant effect of treatment that depended on the pH of the sites was found (MANOVA R square = 0.43 ; treatment:  $F_{(5, 36)} = 8.73$ ,  $p = 0.01$ ; pH:  $F_{(5, 36)} = 8.57$ ,  $p = 0.01$ ; interaction:  $F_{(5, 36)} = 3.53$ ,  $p = 0.01$ ). For each acidity level (acid or neutral streams), no effect of stream was found on the behavioural response of salmon (i.e., no significant difference in response between the acidic streams and likewise, no significant difference in response between the neutral streams;  $F_{(1, 70)} = 0.57$ ;  $p = 0.23$ ).

Under neutral conditions, juvenile Atlantic salmon significantly decreased the time spent

in motion ( $F_{(1, 70)} = 41.56, p = 0.001$ , Figure 1.2a), and the number of feeding attempts ( $F_{(1, 70)} = 30.32, p = 0.010$ ; Figure 1.2b), while the time spent on the substrate and the time spent absent (or hiding) from the stimulus injection location increased ( $F_{(1, 70)} = 17.96, p = 0.08$ ; Figure 1.2c and  $F_{(1, 70)} = 7.46, P = 0.008$ ; Figure 1.2d respectively) when exposed to the conspecific alarm cue compared to stream water. However, no significant difference in the number of aggressive interactions between either treatment was found ( $F_{(1, 70)} = 0.89; p > 0.05$ ). Since the occurrence of such behaviour was rare (mean  $\pm$  standard deviation =  $0.08 \pm 0.015$  interactions per minute), I did not analyze it any further. Under weakly acidic conditions however, there was no significant effect of treatment on the behavioural parameters tested (time spent in motion ( $F_{(1, 70)} = 1.59; p > 0.05$ ; Figure 1.2a); number of feeding attempts ( $F_{(1, 70)} = 2.45; p > 0.05$ , Figure 1.2b); time spent on the substrate ( $F_{(1, 70)} = 0.09; p > 0.05$ ; Figure 1.2c); time spent away from the stimulus injection area ( $F_{(1, 70)} = 0.45; p > 0.05$ ; Figure 1.2d). As a control for potential temporal effects associated with the experimental design, the baseline (pre-stimulus) scores between stream water and alarm cue stimuli treatments for the neutral and acidic sites were compared using multiple one-way ANOVAs. No significant difference was found in any baseline activity scores between the two groups of sites, nor between treatments (multiple one-way ANOVA,  $p > 0.05$ ).

Subsequent one-way ANOVAs revealed a significant difference in response intensity in the alarm cue treatment between parr and YOY in the neutral sites in two of the five behavioural measurements, but none in the weakly acidic streams. Parr spent significantly more time on the substrate than YOY ( $F_{(1, 19)} = 8.727, p < 0.05$ ; Figure 1.2c),

but significantly less time absent (or hiding) from the stimulus injection area than the latter ( $F_{(1, 19)} = 7.34$ ,  $p < 0.05$ ; Figure 1.2d).

## **Discussion**

These data demonstrate that juvenile Atlantic salmon found in the studied acidic streams were impaired in their ability to detect and respond to damage-released chemical alarm cues. The response of individuals to the alarm cues injections did not differ from stream water injections in any of the acidic sites. Also, the age class had no effect on the response intensity in acidic conditions whereas in neutral conditions, differences occurred. Overall, the findings of this experiment are consistent with the results obtained by Leduc et al. (2004a) and support the prediction that even weak levels of acidity directly affect chemosensory risk assessment.

The loss of alarm function in acidic conditions may be due to the two following mechanisms. First, physiological stress and/or olfactory damage may account for the apparent lack of response. I did not find, however, any significant difference in mean baseline activity levels between individuals in the acidic streams and individuals in the neutral streams. Peterson et al. (1989) found that the threshold of pH avoidance for Atlantic salmon is approximately 4.5 while the most acidic condition I found was a pH of 5.71. Hence, the lack of difference in baseline behaviour and the relatively weak acidity level suggest that individuals present in both groups of streams were well suited to live in their habitat and did not experience significant stress linked to acidity. Alternatively, damage to olfactory receptors may account for the lack of response. Leduc et al. (2004b) have shown that YOY rainbow trout could be conditioned to learn a novel odour when



paired with damage-released chemical alarm cues. This learned response however, did not occur when the odour was paired with acidified alarm cues (at pH ~ 6.0). In contrast, acidifying the odour had no effect on the retention of this novel learning as long as the paired alarm cue was not acidified. This result suggests that under weakly acidic conditions, the olfactory function of salmonids is not impaired and thus, cannot explain the lack of alarm response. In the second proposed mechanism, the alarm cue may be completely degraded or the concentration of 'active' alarm cues may be reduced below a detectable level due to ambient pH (Leduc et al. 2003). Several authors have shown that concentrations of alarm cues below some population specific threshold fail to elicit an overt antipredator response (Brown et al. 2001a; Mirza and Chivers 2003; Roh et al. 2004). I do not know the concentration of 'active' alarm cues presented to the test fish therefore I cannot exclude the effect of a covert response (i.e. a non observable response elicited by concentration levels of alarm cues that would be under the minimal concentration threshold to elicit an observable response; Brown et al. 2001b; Mirza and Chivers 2003). Leduc et al. (2004b) have shown that no acquired recognition of a novel odour paired with an alarm cue occurred under weakly acidic conditions while it was shown under neutral conditions. These results suggest that a complete degradation of the alarm cues occurs under acidified conditions.

It is now well established that wild Atlantic salmon populations have steadily been declining over the last three decades (Parrish et al. 1998). The explanation of this decline includes a synergetic interaction of many deleterious effects including invasion of farmed salmon, over-fishing, habitat destruction, pollution (including acidification) and changing oceanic conditions (Parrish et al. 1998; Fleming et al. 2000). While many

salmonid species are suited to live in weakly acidic conditions (Scott and Crossman 1973; Rosseland et al. 2001), there may be an indirect cost linked to increased predation success of predators. It is hypothesized that predation pressure is reduced in acidic conditions with predatory fish absent or reduced due to ambient pH (Bendell and McNicol 1987). I found in both the acidic and neutral streams, several fish species that are known predators of juvenile Atlantic salmon such as brook trout, slimy sculpin (*Cottus gognatus*) and 2+ Atlantic salmon parr (Henderson and Letcher 2003). Moreover, predation pressure on juvenile Atlantic salmon in their nursery streams may come from birds (Wood 1987) and mammals (Heggenes and Borgstrøm 1988). Hence the range of potential predators may not be directly affected by ambient acidity. As such, the observed alarm cue impairment could likely have significant fitness costs linked to increased predation success of juvenile salmon's predators.

Fishes present in an acidic environment could face a significant disadvantage compared to fishes living in neutral conditions, owing to their impaired ability to detect damage-released alarm cues. Responding to alarm cues of both conspecifics and heterospecifics has been shown to increase the survival of prey individuals in encounters with live predators (Mirza and Chivers 2000; Chivers et al. 2002). Likewise, the identity of a novel predator is learned faster from the detection of chemical alarm cues than from visual cues (Brown et al. 1997). This difference in recognition time should be especially valid in a structurally complex environment as often found in many nursery streams used by Atlantic salmon. Hence, sub-lethal effects associated with acidity may exist for juvenile salmon, as for prey fishes in general. Aside from prey fishes, several taxa of aquatic vertebrates and invertebrates including amphibians, arthropods and flatworms

have been shown to respond to chemical alarm cues to mediate predation risk (Kiesecker et al. 1996; Wisenden and Millard 2001). As such, anthropogenic acidification of natural water-bodies may also influence a wide variety of aquatic organisms in their ability to use chemical alarm cues to assess predation risk.

As aquatic waterways may suffer from depleted buffering capacities in many areas affected by acid precipitations (Stoddard et al. 1999; Clair et al. 2004), deleterious effects on juvenile salmonid populations may arise. Therefore, even slight increases in acid precipitation may affect the ability of prey individuals to assess local predation risk via chemosensory cues, in spite of populations' tolerance to acidity. It is of interest to mention that other anthropogenic pollutants affect chemosensory risk assessment in fishes (Scholz et al. 2000; Scott et al. 2003; McPherson et al. 2004). As such, measures to effectively avoid deleterious direct and indirect effects linked to anthropogenic pollution in natural waterways should aim at looking at an integrated approach (Parrish et al. 1998). Changes in the chemistry of aquatic ecosystems may hinder juvenile salmonids from using an ecologically significant source of information on local predation risk. Should this be the case, it may contribute to hindering the recovery of wild Atlantic salmon stock from a depleted state.

Table 1.1. Name and location of the studied sites.

Site	Coordinates
Catamaran Brook	46° 51, 49 N; 66° 09, 54 W
Correy Creek	46° 52, 66 N; 66° 02, 14 W
Devil's Brook 'High'	46° 52, 40 N; 66° 13, 75 W
Devil's Brook 'Low'	46° 52, 37 N; 66° 19, 60 W
Little Southwest Miramichi River	46° 52, 82 N; 66° 05, 99 W
Otter Brook	46° 52, 26 N; 66° 010, 10 W

Table 1.2. Mean ( $\pm$  SE) values for the physical and chemical variables of the studied streams.

	pH	Water Temp. (°C)	Air Temp. (°C)	Current Velocity (cm/s)	Canopy Cover (%)	Cloud Cover (%)	Depth (cm)
Catamaran	7.25	17.01	19.74	52.33	63.09	43.21	37.02
Brook	$\pm 0.07$	$\pm 0.12$	$\pm 0.28$	$\pm 3.53$	$\pm 2.15$	$\pm 2.76$	$\pm 2.21$
L. Southwest	7.13	16.85	20.54	66.06	12.50	29.65	53.70
Miramichi	$\pm 0.17$	$\pm 0.14$	$\pm 0.74$	$\pm 2.26$	$\pm 3.45$	$\pm 2.46$	$\pm 2.32$
Otter Brook	7.02	16.31	19.88	26.66	88.63	28.13	28.30
	$\pm 0.08$	$\pm 0.93$	$\pm 0.86$	$\pm 1.36$	$\pm 1.25$	$\pm 1.78$	$\pm 1.24$
Correy Creek	5.97	17.71	22.50	40.33	74.35	33.86	34.07
	$\pm 0.06$	$\pm 1.20$	$\pm 0.43$	$\pm 2.12$	$\pm 8.23$	$\pm 7.98$	$\pm 1.46$
Devil Brook	5.85	15.92	24.08	40.33	57.99	56.19	28.48
‘High’	$\pm 0.12$	$\pm 0.19$	$\pm 0.47$	$\pm 3.46$	$\pm 7.63$	$\pm 4.32$	$\pm 1.44$
Devil Brook	5.95	18.44	25.23	37.41	66.17	17.38	45.54
‘Low’	$\pm 0.11$	$\pm 0.17$	$\pm 0.42$	$\pm 2.53$	$\pm 8.43$	$\pm 9.51$	$\pm 2.47$

Table 1.3. Mean value ( $\pm$  SE) of the physical and chemical variables between neutral and acidic sites for June and August. One-way ANOVAs were used to assess differences among groups of neutral and acidic streams for the surveyed variables.

June	Neutral	Acidic	F	df	p
Water Temp. ( $^{\circ}$ C)	16.8 $\pm$ 0.07	16.9 $\pm$ 0.14	0.104	1, 69	0.748
Air Temp. ( $^{\circ}$ C)	22.2 $\pm$ 0.07	23.9 $\pm$ 0.28	0.67	1, 69	0.120
Canopy Cover (%)	37.6 $\pm$ 0.41	46.7 $\pm$ 0.40	7.673	1, 69	0.070
Current Vel. (m/s)	0.50 $\pm$ 2.55	0.45 $\pm$ 1.65	0.867	1, 69	0.355
Cloud Cover (%)	26 $\pm$ 19	34 $\pm$ 27	0.403	1, 69	0.528
Depth (m)	0.39 $\pm$ 1.82	0.36 $\pm$ 1.36	0.097	1, 69	0.756
pH	7.14 $\pm$ 0.01	5.88 $\pm$ 0.11	1299.83	1, 69	0.001

August	Neutral	Acidic	F	df	p
Water Temp. ( $^{\circ}$ C)	16.9 $\pm$ 0.09	17.3 $\pm$ 0.12	2.975	1, 69	0.089
Air Temp. ( $^{\circ}$ C)	17.9 $\pm$ 0.18	20.2 $\pm$ 0.45	1.681	1, 69	0.150
Canopy Cover (%)	45.4 $\pm$ 0.04	37.9 $\pm$ 0.59	0.969	1, 69	0.328
Current Vel. (m/s)	0.50 $\pm$ 2.22	0.44 $\pm$ 1.23	1.963	1, 69	0.166
Cloud Cover (%)	33 $\pm$ 16	36 $\pm$ 20	0.337	1, 69	0.564
Depth (m)	0.39 $\pm$ 1.84	0.34 $\pm$ 1.31	1.706	1, 69	0.196
pH	7.16 $\pm$ 0.13	5.87 $\pm$ 0.01	7670.76	1, 69	0.002

Significance was established when  $p \leq 0.05$

Table 1.4. Total area of skin fillets (cm<sup>2</sup>) collected from YOY (0+) and Parr (1+) Atlantic salmon and the volume of water added to generate the chemical alarm cues.

Year-class	Skin area (cm <sup>2</sup> )	Water volume (mL)	Dilution (cm <sup>2</sup> · mL <sup>-1</sup> )
Parr (June)	327.74	2173.73	0.1508
Parr (August)	348.21	2198.32	0.1583
YOY (June)	332.17	2199.40	0.1510
YOY (August)	319.80	2132.13	0.1502

Figure 1.1. The location of the study sites in New Brunswick, Canada. The inset map shows the location of New Brunswick within Canada (shaded area) as well as the location of the study sites within the province of New Brunswick. The numbers 1 to 6 correspond to study sites (each approximately 50 m in length) located on Catamaran Brook, Otter Brook, Little Southwest Miramichi River, Devil's Brook 'High', Devil's Brook 'Low' and Correy Creek, respectively.



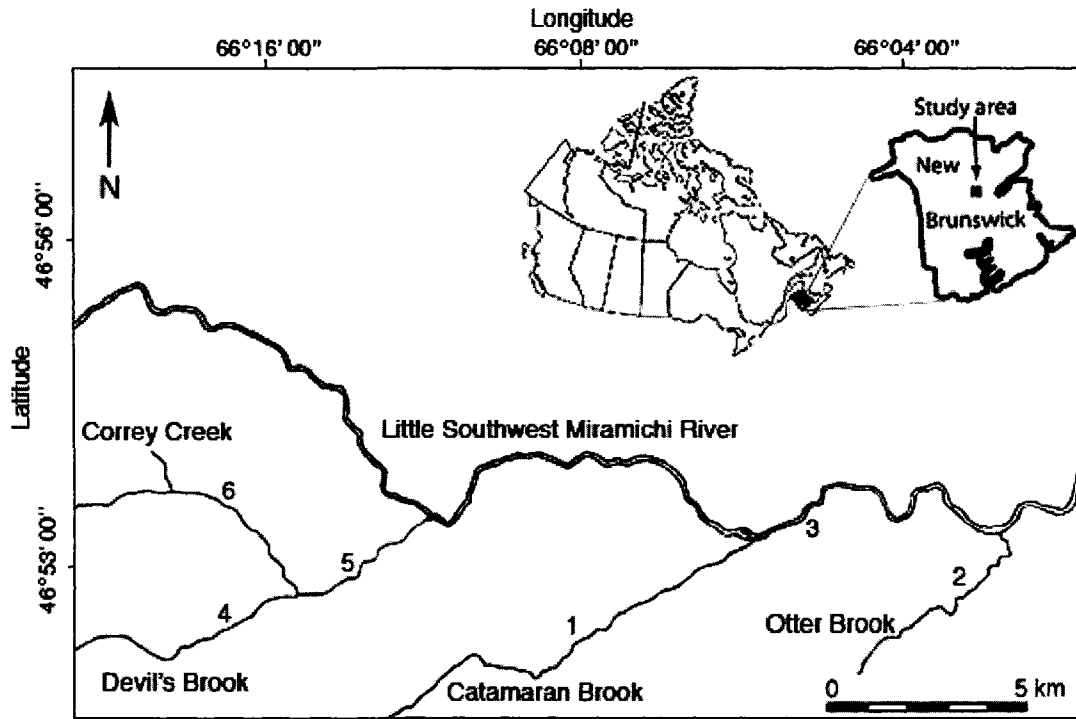
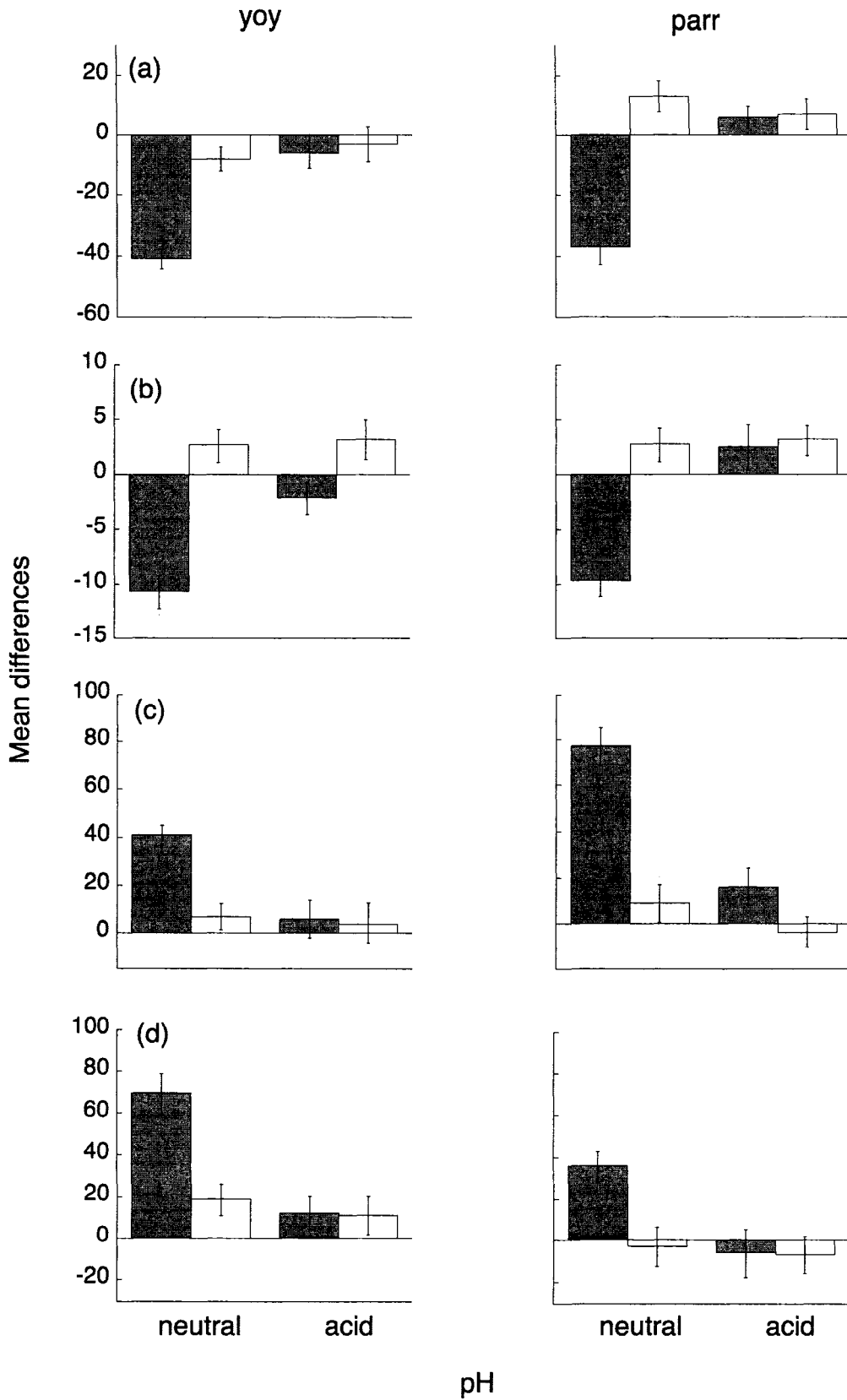


Figure 1.2. Mean ( $\pm$  standard error) differences (post - pre) in: a) time in seconds spent in motion, b) number of feeding attempts, c) time in seconds spent on the substrate and d) time spent in seconds away or hiding for juvenile Atlantic salmon (*Salmo salar*) exposed to either alarm cues (dark bars) or stream water (open bars) under neutral (pH  $\sim$  7.0) or acidic (pH  $\sim$  6.0) conditions for YOY (left column) and parr (right column).



Stimuli and acidity treatments

**Chapter 2. Antipredator responses of wild juvenile Atlantic salmon (*Salmo salar*) to chemical alarm cues: comparing the effects of environmental acidity versus population differences.**

**Introduction**

Various chemosensory functions may be affected by anthropogenic changes in water chemistry (Lürling and Scheffer 2007). In various fish taxa, sub-optimal behavioural responses have been shown to occur following exposures to pesticides (Atchison et al. 1987; Little et al. 1990; Scholz et al. 2000), heavy metals (Scott et al. 2003; McPherson et al. 2004), polychlorinated biphenyls (Lürling and Scheffer 2007) and acidification (Åtland 1998; Brown et al. 2002; Leduc et al. 2004a). In their experiments, Leduc et al. (2004a, 2006) showed that, in neutral nursery streams (pH range of ~7.0 – 7.4), conspecific alarm cues elicited a measurable increase in alarm response in juvenile wild brook trout (*Salvelinus fontinalis*) and Atlantic salmon (*Salmo salar*). However, when tested in weakly acidic streams (pH range ~ 5.8 – 6.2), these salmonids did not display any measurable alarm response. These results suggest that ambient acidification is creating an environmental constraint on this chemosensory alarm function. Given the demonstrated importance of chemosensory cues in the mediation of threat-sensitive decisions in the detection and avoidance of predators (see General Introduction and Chapter 1), it is likely that any impairment from acidification could have significant consequences on juvenile Atlantic salmon.

Alternatively, chemosensory abilities may vary across populations if differences in alarm cue detection or production exist. First, the apparent loss of chemosensory

alarm function may be independent of environmental acidity. Initially, differences in olfactory and gustatory sensitivity between fish populations exist, leading to inconsistent abilities to detect water-borne cues (Hara 1999). Consequently, the ability to detect and respond to chemical alarm cues may be affected by intrinsic sensory sensitivity differences rather than resulting from an environmental disturbance. Second, the quantity and/or quality of the chemical alarm cues produced by prey fishes may be affected by individual body state. For example, compared to poorly fed alarm cue donors (the sender of the chemical alarm cues), well-fed donors produced chemical alarm cues eliciting a significantly greater intensity of antipredator behaviour in the receiving individuals (Brown et al. 2004). Thus, it is not known if the observed impairment of the ability of prey fish to detect and respond to chemical alarm cues is affected by differences between populations in the production and/or detection of the chemical alarm cues.

In this study I assessed the contribution of environmental acidity versus potential population effects on the antipredator response in juvenile wild Atlantic salmon exposed to conspecific chemical alarm cues. I related this response to the effects of ‘environment’ and ‘populations’ to determine if the intensity of the antipredator response was best explained by environmental or population effects, or a mixture of both. To address these questions, I conducted two experiments. In the first, I conducted a reverse transplant between fish initially found under neutral and weakly acidic habitats and assessed whether environmental or population differences would better explain for the occurrence of antipredator behaviour. In the second experiment, I conducted exposures of chemical alarm cues of different salmon populations as well as a sympatric heterospecific prey-guild member species under neutral conditions. This was done in order to assess whether

either of these populations produced chemical alarm cues having the potential to elicit antipredator behaviour of similar intensity in a receiving population.

## **Materials and methods**

### **EXPERIMENT 1**

The purpose of this reciprocal transplant experiment was to measure the antipredator response to a standard alarm cue of juvenile Atlantic salmon from habitats differing in ambient pH. Salmon from a weakly acidic habitat were tested in a weakly acidic or a neutral stream enclosure. Likewise, salmon from a neutral habitat were tested in a weakly acidic or neutral stream enclosure.

#### *Test sites*

I conducted this experiment between June 27<sup>th</sup> and July 21<sup>st</sup> 2006 in Northumberland County, New Brunswick, Canada in two tributaries of the Little Southwest Miramichi River, Catamaran Brook and Devil's Brook (Figure 2.1; for coordinates, see Table 1.1). Catamaran Brook and Devil's Brook are natural nursery streams used by wild Atlantic salmon located in mature forests suffering little or no direct human disturbance effects, aside from potential acid precipitations (Cunjak 1993; A.O.H.C. Leduc, Concordia University, unpublished data). A great portion of the region is underlain by poorly weatherable bedrock including granite with little acid buffering capacity (Department of Energy, Mines and Resources Canada, 1991). At the time of the experiment, these streams differed in their mean acidity level (Catamaran Brook, pH range of 7.19 – 7.38; Devil's Brook, pH range 6.01 – 6.19; Table 2.1). According to Leduc et al. (2006,

2007b) these pH conditions have been stable since 2003 in these streams suggesting that the occurrence of such ambient acidity is persistent. Each site was 30 m long and were chosen to be similar in their physical attributes (Table 2.1). In these sites, enclosures were built by fencing off individual channel units, using 4.5 mm wire mesh supported by steel bars (as in Rosenfeld and Boss 2001). Enclosures were 6 m long, 1 m wide and 0.7 m high and installed parallel to the water current, with a bottom skirt stapled to a 5 cm x 10 cm x 60 cm wooden plank (“two-by-four”) buried under the substratum. To prevent fish from escaping, I used a mesh bottom that I covered with gravel and cobbles (to imitate the natural substratum). To reduce the possibility of transmission of visual alarm cues (see Mathis et al. 1996) between test salmon and to reduce intraspecific aggressive interactions (Imre et al. 2002; Blanchet et al. 2006), I placed 30 - 40 cm high divisions, consisting of natural boulders, in the enclosures, creating six visually isolated ‘compartments’ (one division every meter). The enclosures were left for a minimum of 48 hours before conducting any behavioural trial (see below).

### *Stimulus types*

On June 27<sup>th</sup>, using a backpack electrofisher unit (Smith-Root Electrofishing Co., Vancouver, WA), I captured 16 young-of-the-year (YOY) Atlantic salmon (mean  $\pm$  SD fork length = 36.0 mm  $\pm$  2.3) from the Little Southwest Miramichi River (Figure 2.1). I homogenized a total of 79.6 cm<sup>2</sup> of skin fillets harvested from their bodies (see Chapter 1, Material and methods). The resulting solution had a pH of approximately 7.1. I used donors from the Little Southwest Miramichi River population to produce a ‘standard’ chemical alarm cue solution used at both Catamaran Brook and Devil’s Brook sites. As a

control stimulus for the injection of a fluid, I used stream water (from the test stream). Both the control and the experimental stimuli were packaged into 15 mL aliquots and frozen at  $-20^{\circ}\text{C}$  until needed.

### ***Experimental protocol***

I conducted direct behavioural observation trials on focal test fish placed inside an enclosure using a 2 x 2 x 2 design with ‘enclosure location’, ‘test fish origin’ and ‘stimuli’ as factors. At least 24 hours before each test trial (ranging from 24 to 27 hours), I placed six YOY salmon in both enclosures at a density of 1 individual per square meter, a density naturally found in these streams (Steingrímsson and Grant 2003). I randomly chose the origin of the test fish (from either Catamaran Brook or Devil’s Brook) to be placed in a given enclosure (in either the Catamaran Brook or Devil’s Brook’s enclosure). The test subjects were captured using dip-nets while snorkelling outside of the study sites (minimum of 50 m away from the test sites). In this experiment, the test fish were placed inside the enclosure while I was positioned outside to observe. Prior to each trial, I let the focal fish acclimate to my presence until it behaved ‘normally’ (i.e., it was feeding and moving; Dill and Fraser 1984). Observation trials were 10 minutes in duration and divided in two blocks of 5 minutes. These blocks corresponded to the pre- and the post-stimulus periods and were separated by the injection of one of the 2 stimulus types (either alarm cue or stream water, randomly chosen) using a 60 mL syringe handled by the observer. Stimuli were injected from outside of the enclosure (through the mesh) from approximately 0.5 m upstream from the focal fish. Behavioural observations were directly recorded using a water-resistant stopwatch and a counter-clicker. To avoid



exposing the test fish to multiple injected stimuli, I conducted the observations from the most downstream fish to the most upstream. Typically, only one fish was present in a visually isolated compartment (see above). When more than one fish were present in a compartment, I randomly chose one fish for observations. After each trial, I measured the pH, enclosure depth, current speed, dissolved oxygen, water conductivity, percent cloud cover and water temperature (Table 2.1) to account for possible micro-habitat differences that might account for any observed trends. The current velocity was recorded from 5 cm under the water surface using a Flo-Mate velocity meter (Marsh McBirney Inc., Frederick, MD). I measured the pH, dissolved oxygen, conductivity and water temperature, using a WTW-P 4 MultiLine meter (WTW, Weilheim, Germany) and the depth using a one-meter ruler.

Because of the limited number of enclosures (one in each stream), experimental trials were run sequentially for 20 days. I conducted 12 replicates per combination of treatments for a total of 96 observation trials (12 replicates x 2 fish origins (Catamaran or Devil's Brook) x 2 enclosure locations (neutral or acidic stream) x 2 stimulus types (chemical alarm cues or stream water)). As in Chapter 1, I conducted dye injection tests using 2% commercial milk to ensure that the injected stimuli reached the focal fish. After the experimental trials were completed, the test fish were released at the location of their capture.

### ***Behavioural measures and statistical analysis***

To quantify the intensity of an alarm response, I measured the time (in seconds) spent in movement and the number of feeding attempts during the pre- and post-stimulus

observation periods (as in Leduc et al. 2004a, 2006, 2007a). The time spent in movement was measured following any observable displacement exceeding one body length while a feeding attempt included a displacement of at least half a body length followed by a pecking motion. The pre-stimulus values were subtracted from the post-stimulus values to give me a difference score for the measured behavioural parameters (see above). I assumed that a reduction in feeding and movement indicated an antipredator response (Chivers and Smith 1998). I tested for any overall effect of enclosure location (neutral versus acidic environment), test fish origin (Catamaran Brook or Devil's Brook) and stimulus type (stream water versus alarm cue) on the antipredator behaviour intensity of test fish, using a multivariate analysis of variance (MANOVA) with stimulus, environment and origin of the fish as independent variables. Using subsequent MANOVAs, I further investigated the effects of stimulus and origin of the fish on the antipredator response intensity of the test fish for each stream separately.

To determine whether the behavioural responses were the product of differences in habitat characteristics between streams, I analyzed the physical and chemical data using an analysis of variance (ANOVA; Table 2.1). To reduce the probability that the responses were the product of differences associated with the test fish populations, I compared the baseline (pre-stimulus) values of each behavioural measure between the two studied streams and populations using subsequent ANOVAs. The data respected conditions of equality of the variances. I used SPSS 11 to conduct all the statistical analysis.

## EXPERIMENT 2

The purpose of this experiment was to assess whether the ‘potential’ to trigger an alarm response to chemical alarm cues is influenced by the pH of the environment of the donor condition. To do this, I assessed the antipredator response of juvenile salmon found under neutral conditions exposed to the chemical alarm cues collected from donors from different ambient pHs.

### *Test sites*

I conducted this experiment in Catamaran Brook (for coordinates, see Chapter 1, Table 1.1), a third order tributary of the Little Southwest Miramichi River (Figure 2.1). I chose an approximately 200 m long section of Catamaran Brook (Figure 2.1) having homogeneous physical and chemical characteristics (Table 2.2).

### *Stimulus collection*

To conduct this experiment, I used chemical alarm cues from three different populations of juvenile Atlantic salmon (see below) and from blacknose dace (*Rhinichthys atratulus*) (a species sympatric with Atlantic salmon). Using a backpack electrofisher unit, I captured juvenile Atlantic salmon from Catamaran Brook, Devil’s Brook and the Little Southwest Miramichi River (Figure 2.1) and prepared the chemical alarm cues in the same fashion as in Experiment 1. I prepared chemical alarm cues using the same skin area per volume of water (Table 2.3) for these different salmon populations and stored them in aliquots of 20 mL at  $-20\text{ }^{\circ}\text{C}$  until needed. In an identical fashion, I generated chemical alarm cues from blacknose dace that were captured from the Catamaran Brook

site, as this species shares common predators with juvenile salmon (A.O.H.C. Leduc, Concordia University, personal observations). Several experiments have demonstrated that prey individuals can learn to detect and respond to chemical alarm cues from other species when they live in sympatry (heterospecific response; Chivers et al. 2002; Brown and Chivers 2005) and that such antipredator response to heterospecific cues may confer increased survival benefits (Mirza and Chivers 2002). I, therefore, included the heterospecific alarm cue from blacknose dace to compare it with conspecific salmon alarm cues originating from different sites. As a control for the injection procedure, I used unchlorinated well water.

If no difference existed in the quality and/or quantity of the chemical alarm cues produced by the three salmon populations sampled, I predicted that an exposure to any of these chemical alarm cues should elicit antipredator responses of similar intensity (see below), whereas, if the quality or quantity of chemical alarm cues produced is linked to a site effect or a population effect, the alarm cues from both salmon and blacknose dace from the test site would elicit a significantly greater response than those from fish from the two 'out-of-site' populations (i.e., Devil's Brook and Little Southwest Miramichi River).

### ***Experimental protocol***

Trials were conducted as in Chapter 1, by moving from downstream to upstream and spacing trial sites by at least 4 m. The territory size of YOY salmon typically ranges from 1.5 – 3 m<sup>2</sup> (Steingrímsson and Grant 2003). I conducted 15 replicates for each stimulus type for a total of 75 trials (5 stimuli x 15 trials).

As before, I measured physical and chemical variables after each trial to assess whether the environmental conditions could have had affected the salmon's alarm response (Table 2.2). In addition, I measured the substrate complexity at the focal location of each test fish (i.e. the location where they received the stimulus) using a one-meter long flexible metal wire that I moulded to the substrate. I obtained a value by measuring the linear distance between the ends of the wire after it was moulded to the substrate and dividing this distance by the original length of the wire. Since the metal wire had a length of one meter, a substrate complexity value of 'one' would indicate that the substrate is flat (1 m divided by 1m; i.e., no complexity) while a lower number would indicate greater complexity. I took this measure in the direction of the water current and perpendicular to it and averaged these two values (Table 2.2).

### ***Behavioural measures and statistical analysis***

To assess for the occurrence of antipredator behaviour, I recorded the number of feeding attempts and the time (in seconds) spent in motion as in Experiment 1. In addition, I quantified the time spent on the substrate (in seconds) measured as the time a focal fish stayed in physical contact with the substratum without changing its location.

I compared the change in antipredator response intensity for each measured behaviour between the injections of the different chemical alarm cues against well-water injections (control) using a univariate analysis of variance. I also used univariate analysis of variance with simple contrast, to compare the intensities of the alarm responses between the different treatments to the 'in-site' salmon alarm cue treatment. I used SPSS 11 to conduct the statistical analysis

## Results

### EXPERIMENT 1

There was a significant overall effect of enclosure location (Catamaran Brook (neutral) versus Devil's Brook (acidic);  $F_{2, 87} = 5.10$ ,  $p < 0.01$ ) as well as a significant interaction between stimulus type (alarm cue versus stream water) and enclosure location ( $F_{2, 87} = 8.52$ ,  $p < 0.001$ ) on the alarm response intensity of juvenile salmon. No significant effect of test fish origin (from Catamaran Brook or Devil's Brook) or stimulus type existed ( $F_{2, 87} = 0.53$ ,  $p = 0.546$ ) nor an interaction between the two ( $F_{2, 87} = 0.034$ ,  $p = 0.966$ ). For fish tested in the Catamaran Brook enclosure (neutral condition), a significant effect of stimulus type used existed ( $F_{2, 43} = 8.52$ ,  $p = 0.001$ ) while the origin of test fish had no effect on the intensity of the alarm response ( $F_{2, 43} = 0.56$ ,  $p = 0.351$ ) nor an interaction between origin and stimulus ( $F_{2, 43} = 0.096$ ,  $p = 0.909$ ). Under such neutral conditions, following exposures to the alarm cues salmon of both origins significantly reduced their feeding attempts ( $F_{1, 44} = 12.26$ ,  $p = 0.001$ , Figure 2.2a) and their time spent moving ( $F_{1, 44} = 6.53$ ,  $p = 0.042$ , Figure 2.2b) compared to the injection of stream water. In Devil's Brook (acidic conditions), I found no significant effect of the stimuli used ( $F_{2, 43} = 0.83$ ,  $p = 0.910$ ) or the origin of test fish ( $F_{2, 43} = 1.12$ ,  $p = 0.334$ ) or no interaction between the two ( $F_{2, 43} = 0.076$ ,  $p = 0.927$ ). I found no significant difference in the number of feeding attempts ( $F_{1, 44} = 0.16$ ,  $p = 0.960$ ; Figure 2.2a) or the time spent in motion ( $F_{1, 44} = 0.19$ ,  $p = 0.849$ ; Figure 2.2b).

To ensure that these results were not due to differences in baseline activity levels between the test fish's origin or the testing location, I compared their activity rates before the injection of a stimulus for each behavioural measure using a MANOVA with

'enclosure location' and 'fish origin' as independent variables. I found no significant difference in overall baseline activity between fish studied in the two test streams ( $F_{2, 91} = 2.084$ ,  $p = 0.130$ ), between the origin of the test fish ( $F_{2, 91} = 0.781$ ,  $p = 0.456$ ), nor an interaction of streams x origin ( $F_{2, 91} = 0.063$ ,  $p = 0.938$ ).

To further ensure that the behavioural response differences measured were not the product of differences associated with the environmental conditions between both enclosures, I compared the abiotic variables found in the enclosures using ANOVAs. With the exception of water pH and conductivity, no significant difference in the mean values for each abiotic variable surveyed existed between the enclosures (Table 2.1).

## EXPERIMENT 2

Univariate analysis of variance revealed significant effects of stimulus type on the frequency of foraging attempts ( $F_{4, 64} = 5.098$ ,  $p = 0.001$ ), time moving ( $F_{4, 64} = 4.983$ ,  $p = 0.01$ ) and time on substrate ( $F_{4, 64} = 3.560$ ,  $p = 0.050$ ). Planned (*apriori*) contrasts revealed that significant differences existed on the intensity of antipredator behaviour of juvenile salmon exposed to any of the salmon chemical alarm cues compared to a control of well-water. No significant difference in alarm response intensity between salmon exposed to the three salmon chemical alarm cues was found suggesting that any salmon alarm cue had the 'potential' to elicit an alarm response. For instance, injections of the alarm cues from any of the salmon populations triggered a significant reduction in feeding attempts (Figure 2.3a), time spent in motion (Figure 2.3b) and a significant increase in time spent motionless on the substrate (Figure 2.3c) compared to a control of water. Interestingly, blacknose dace alarm cues elicited a significant decrease in feeding

attempts compared to a control of water but not significantly different from salmon alarm cues (Figure 2.3a). This trend was not consistent with the other behaviour measured as no significant difference existed between dace alarm cues and water for the time spent moving and the time spent motionless on the substrate (Figure 2.3b and 2.3c, respectively), suggesting that the heterospecific chemical cues elicited a weaker antipredator response than did conspecific cues.

### **Discussion**

These results demonstrate that the ability to detect and respond to waterborne chemical alarm cues is impaired by even weakly acidic conditions. They also show that population differences did not influence the ability to elicit, detect or respond to damage-released chemical alarm cues. The reverse transplant experiment showed that the origin of the test fish did not have any effect on the intensity of the antipredator response following an exposure to the alarm cues while the environmental conditions in which the test fish were present had a direct effect on the intensity of the response (Figure 2.2). When present under neutral conditions, both the test fish from neutral and weakly acidic streams performed predicted antipredator behaviour. By contrast, when tested under weakly acidic conditions, fish from neither population responded to the conspecific chemical alarm cues. When comparing all the measured environmental variables between test sites, only the environmental acidity and conductivity levels significantly differed. Conductivity may represent the contribution of ion-rich ground water coming into the stream leading to greater buffering capacity hence, circum-neutral conditions (Woessner 2000).



In the second experiment, I observed that the quantity and/or quality of the alarm cues produced in the epidermis of prey fish did not significantly differ between salmon populations as a consistent antipredator behaviour was measured following the introduction of these different salmon populations' alarm cues. Juvenile salmon consistently responded more intensely to salmon chemical alarm cues from all salmon populations than to 'in-site' heterospecific blacknose dace alarm cues, although blacknose dace did partially trigger an alarm response (Figure 2.3). Consequently, the differences in antipredator behaviour found between neutral and weakly acidic streams is not likely accounted by significant differences in alarm cues quantity/quality found in the epidermis of these different salmon populations. Rather, I suggest that an environmental difference in acidity level from neutral to weakly acidic directly disrupts chemosensory alarm functions of the chemical alarm cues.

Brown et al. (2002) suggest that a chemical change to the alarm cues occurs under weak acidification, rendering the alarm cues irreversibly non-functional. In their experiment, they sequentially exposed two species of cyprinids to conspecific chemical alarm cues. When the test fish were exposed to alarm cues under neutral pH, a reduction in moving, feeding and area use occurred. However, when these same test fish were re-tested 48h later under weakly acidic conditions (pH 6.0), these typical behavioural changes were no longer detected. Finally, when retested 48 hours later under neutral conditions, the alarm cues exposures translated again into normal alarm behaviour. These findings suggest that no severe olfactory and/or receptor damage could account for the loss of response but rather, a chemical change in the chemical alarm cues occurred under sub-lethal acidic conditions (Brown et al. 2002).

Such acidic conditions (pH range of 6.0 – 6.3) are argued to be above a threshold at which, damage to the aquatic biota occurs (Doka et al. 2003; Holt et al. 2003) and typically, do not create physiological stress in many freshwater fish species including salmonids (Lacroix et al. 1985; Gunn 1986; Peterson et al. 1989). Nevertheless, the observed impairment of normal alarm function may have severe fitness consequences for wild juvenile salmonids. Laboratory and semi-natural enclosure experiments have shown a direct fitness benefit from the ability to detect and respond to conspecific and heterospecific chemical alarm cues. For example, during staged-encounters between prey and live predators, individuals having the ability to rely on these alarm cues survived longer and in greater proportion than ‘naïve’ individuals (Mirza and Chivers 2000, 2001b, 2003). Relating these findings to natural conditions would strongly suggest that prey fish deprived of this sensory modality would suffer a greater predation cost linked to an increased success of their predators. In their experiment, Henderson and Letcher (2003) showed that after the introduction into natural streams of predator-naïve hatchery reared juvenile Atlantic salmon, predators were successful within the first week at preying upon over 60 percent of the introduced salmon fry. After this critical period, the success of predators was greatly reduced. These authors suggested that the survival from this critical period may be a direct correlate of over-summer survival for these juvenile fish. Henderson and Letcher (2003) identified slimy sculpins (*Cottus cognatus*) and brook trout as active predators of salmon fry. Within the study streams, I commonly found these fish species (in addition to avian and mammalian predators), supporting the idea that a predation pressure exists on salmon fry. Under a chemosensory alarm function loss, prey may suffer increased survival cost from predator increased success.

Antipredator behaviour aside, other behavioural components among aquatic organisms can be negatively affected by sub-lethal acidification. For example, Kitamura and Ikuta (2000, 2001) have shown suppressed normal mating, nest digging and migratory behaviour in wild hime salmon (land-locked sockeye salmon, *Oncorhynchus nerka*) under a pH value of approximately 6.2. These authors speculated that the behaviour studied were reduced or stopped as an adaptation to environmental acidic conditions that would not be suitable for developing fry present in their redds (gravel nests). However, as the acidity level in the interstitial substratum space of redds does not fluctuate as much as the outside environment (Gunn 1986), it suggests that most of the deleterious impacts of sub-lethal acidity would occur after fry emerge from their redds, notably, when they are extremely vulnerable to predation.

Changes in certain fish behaviour are acute indicators of individuals' sensitivity to anthropogenic acidity that may exacerbate the effects of natural acidity sources (Gorham et al. 1986; Guerold 2000; Dangles et al. 2004). For instance, in the study system, the acidification sources are likely to be an interaction between natural sources of acidity and anthropogenic acid precipitation. Leduc et al. (2006) found the presence of marshy areas upstream of four weakly acidic streams allowing for acidity to occur through the release of high levels of natural organic acids (Collier et al. 1990; Dangles et al. 2004). Moreover, the Canadian Atlantic provinces are considered in the path of acid precipitations (Doka et al. 2003). Although recent research has reported a widespread aquatic recovery from acidification in North American and European aquatic ecosystems in response to a decrease in sulphate deposition (Stoddard et al. 1999; Doka et al. 2003; but see Alewell et al. 2000), several estimates predict that 50 or even 100 years will be

necessary for the acid neutralizing capacity to return to pre-acidification levels (Jeffries et al. 2000). Stoddard et al. (1999) suggest that a larger decrease in sulphur deposition and/or a longer response time may be required for a widespread recovery to occur in North America. As such, the occurrence of sub-lethal acidity and its impacts on aquatic ecosystems will likely be measurable for many more decades. Although various anthropogenic pollutants may have deleterious effects on aquatic biota, acid precipitation are of particular concern to ecosystems not directly affected by human activities as their harmful impacts (reviewed in Schindler 1988) can be observed at great distances from the point source of their emissions (Rodhe et al. 1995), increasing the difficulty to protect biota.

Table 2.1. Mean value ( $\pm$  standard error) of the physical and chemical variables for the single enclosure in each of Catamaran Brook and Devil's Book. One-way analysis of variance (ANOVA) was used to assess differences between these brooks for the surveyed variables.

	Catamaran Brook	Devil's Brook	F	df	P
pH	7.25 $\pm$ 0.08	6.08 $\pm$ 0.05	15.41	1, 95	0.001
Conductivity ( $\mu$ S/cm)	92.2 $\pm$ 0.13	23.7 $\pm$ 0.07	4930.70	1, 95	0.000
Dissolved oxygen (%)	96.8 $\pm$ 0.07	95.8 $\pm$ 0.12	0.13	1, 95	0.712
Water temp. ( $^{\circ}$ C)	16.6 $\pm$ 0.11	16.4 $\pm$ 0.09	1.62	1, 95	0.205
Current vel. ( $m \cdot s^{-1}$ )	0.23 $\pm$ 0.17	0.21 $\pm$ 0.26	0.59	1, 95	0.444
Depth (m)	0.20 $\pm$ 0.21	0.23 $\pm$ 0.10	3.74	1, 95	0.065
Cloud cover (%)	55 $\pm$ 10	34 $\pm$ 28	0.06	1, 95	0.797
Substrate complexity	0.88 $\pm$ 0.7	0.82 $\pm$ 0.13	0.74	1, 95	0.059

Significance was established when  $p \leq 0.05$ .

Table 2.2. Mean value ( $\pm$  standard error) of the physical and chemical variables for the observation trials of the different stimuli treatments. The three salmon chemical alarm cues are indicated by their stream of origin.

	Catamaran Brook	Little Southwest Miramichi River	Devil's Brook	Blacknose Dace	Water
pH	7.29 $\pm$ 0.06	7.27 $\pm$ 0.09	6.22 $\pm$ 0.03	7.34 $\pm$ 0.08	7.35 $\pm$ 0.02
Dissolved O <sub>2</sub> (%)	98.8 $\pm$ 0.14	97.8 $\pm$ 0.09	96.2 $\pm$ 0.11	96.9 $\pm$ 0.05	98.1 $\pm$ 0.03
Water temp. (°C)	17.0 $\pm$ 0.06	17.2 $\pm$ 0.12	17.6 $\pm$ 0.07	17.4 $\pm$ 0.08	17.8 $\pm$ 0.11
Current vel. (m•s <sup>-1</sup> )	0.26 $\pm$ 0.13	0.33 $\pm$ 0.12	0.33 $\pm$ 0.06	0.28 $\pm$ 0.24	0.27 $\pm$ 0.20
Depth (m)	0.23 $\pm$ 0.22	0.29 $\pm$ 0.26	0.25 $\pm$ 0.31	0.26 $\pm$ 0.19	0.28 $\pm$ 0.73
Cloud cover (%)	52 $\pm$ 14	55 $\pm$ 09	50 $\pm$ 12	61 $\pm$ 10	48 $\pm$ 19
Substrate complexity	0.82 $\pm$ 0.7	0.83 $\pm$ 0.9	0.90 $\pm$ 0.2	0.88 $\pm$ 0.5	0.84 $\pm$ 0.1

Table 2.3. Number of skin donors, donors' mean standard length and total area of skin fillets (cm<sup>2</sup>) collected to generate the chemical alarm cues for each population of juvenile Atlantic salmon (*Salmo salar*) and blacknose dace (*Rhinichthys atratulus*).

Site	No. of donors per site	Mean SL ± SD (mm)	Area of skin collected (cm <sup>2</sup> )	Volume of water added (mL)	Ratio skin-water (cm <sup>2</sup> • mL <sup>-1</sup> )
Catamaran Brook	5	66.2 ± 3.4	32.7	367	0.089
Devil's Brook	6	66.0 ± 2.7	35.8	401	0.089
Little Southwest Miramichi River	12	63.7 ± 2.4	57.1	658	0.087
Blacknose dace	7	55.0 ± 1.4	28.6	323	0.088

Figure 2.1. The location of the study streams in New Brunswick, Canada used for Experiments 1 and 2. The inset map in the top right corner shows the location of the study sites within New Brunswick. Experiment 1: The arrows correspond to the location of the study sites containing the enclosures (each site approximately 30 m in length) located in Catamaran Brook and Devil's Brook. Experiment 2: The asterisk corresponds to the location of the study site in Catamaran Brook. The numbers 1 – 4 correspond to the different fish populations from which damage-released chemical alarm cues were generated (Catamaran Brook, Little Southwest Miramichi River, Devil's Brook and Blacknose dace, respectively).



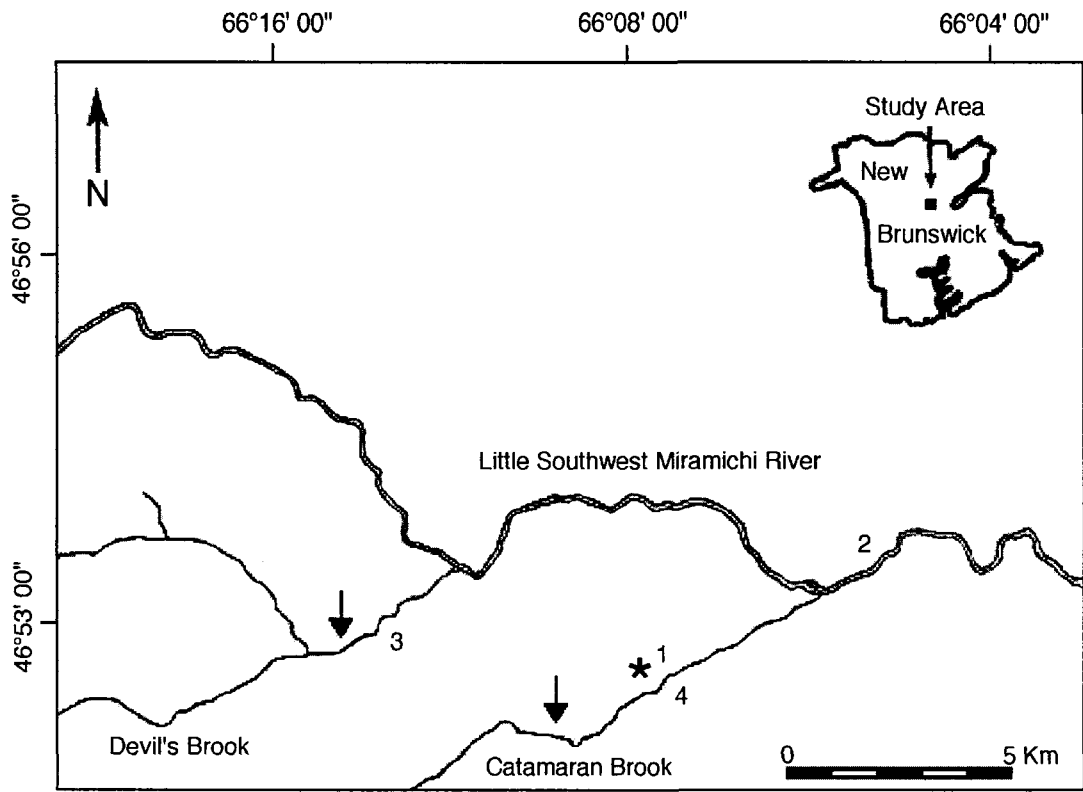


Figure 2.2. Mean ( $\pm$  standard error) differences (post - pre) in: a) number of feeding attempts and b) time in seconds spent in motion for juvenile Atlantic salmon (*Salmo salar*) exposed to either alarm cues (dark bars) or stream water (open bars) under neutral (Catamaran Brook) or acidic (Devil's Brook) conditions. The origin of the test fish is designated by the letters CB (Catamaran Brook) or DB (Devil's Brook).

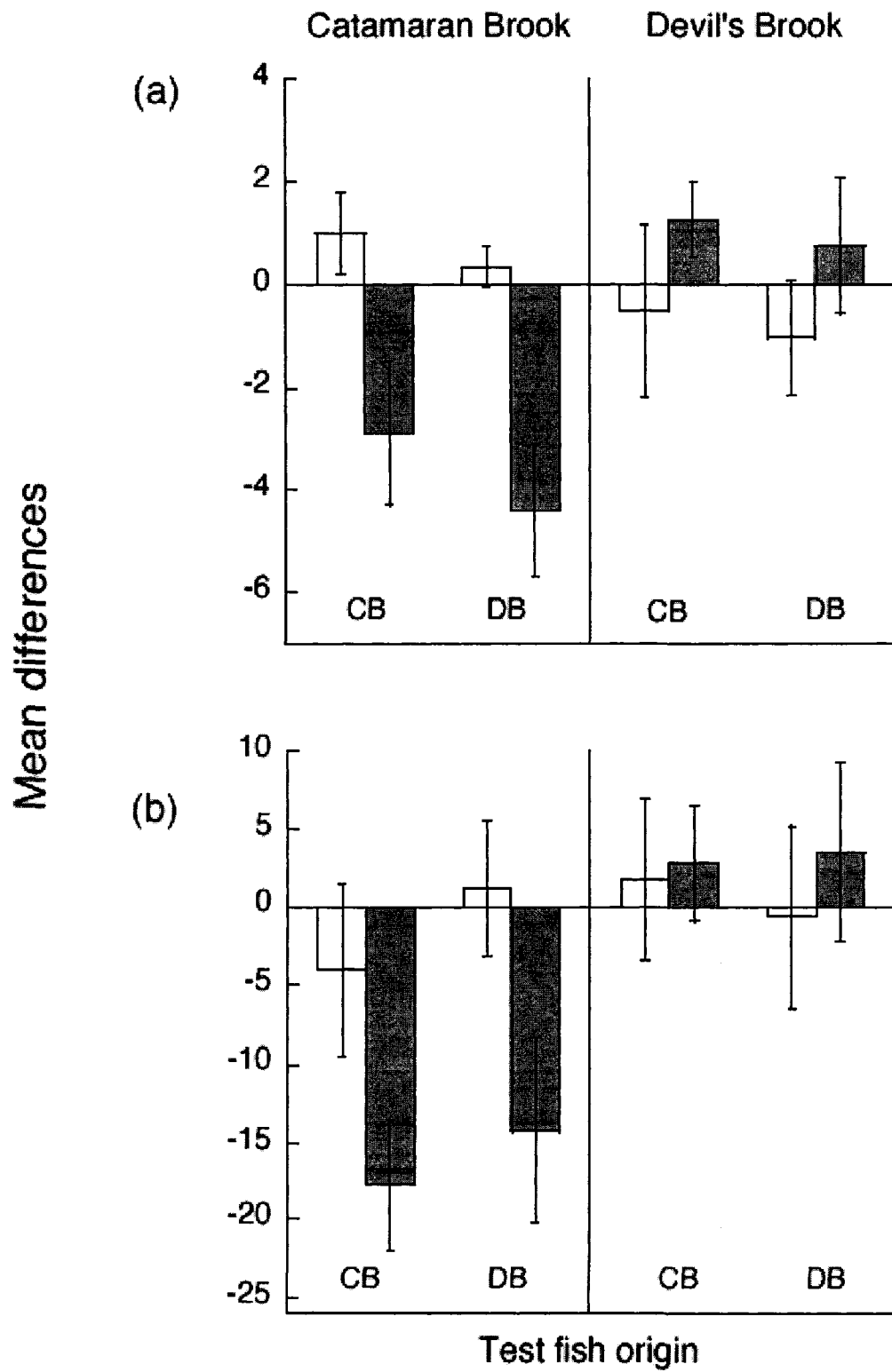
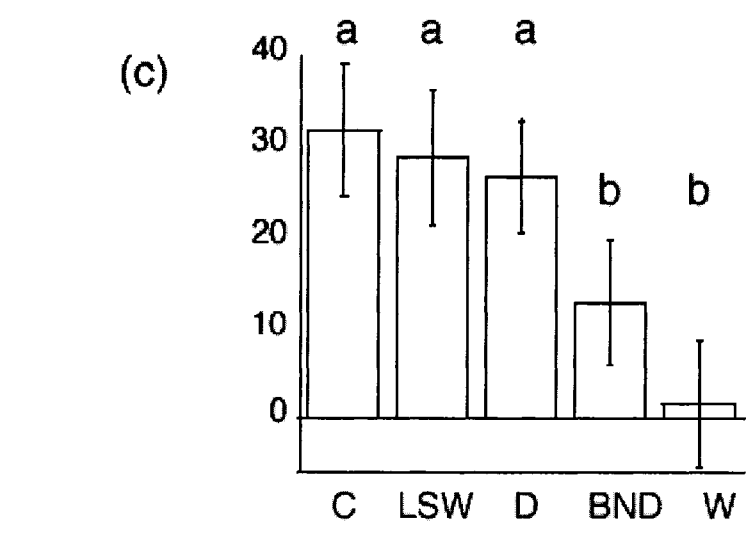
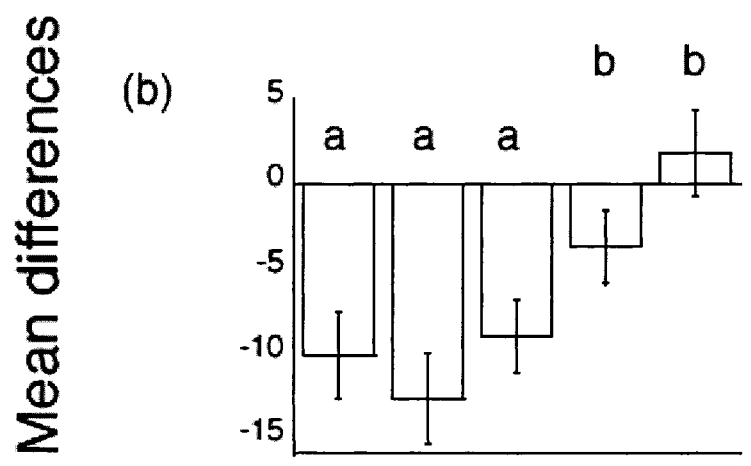
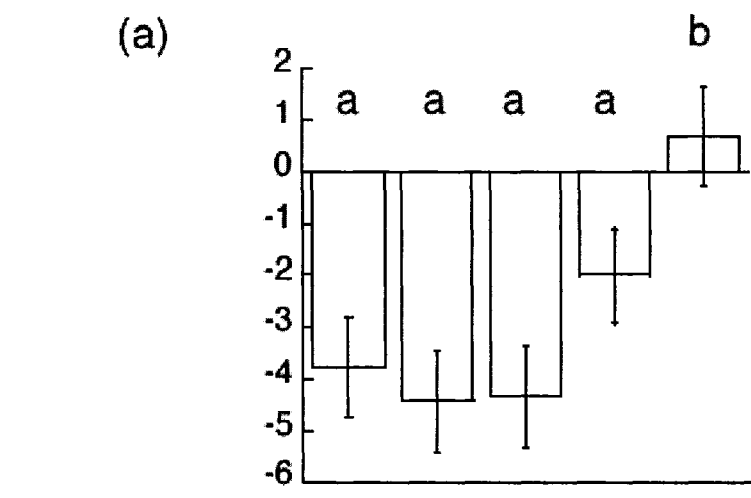


Figure 2.3. Mean ( $\pm$  standard error) differences (post - pre) in: a) number of feeding attempts, b) time in seconds spent in motion and c) time in seconds spent motionless on the substrate for juvenile Atlantic salmon (*Salmo salar*) exposed to the chemical alarm cues of different origins. Catamaran Brook, Little Southwest Miramichi River, Devil's Brook, Blacknose dace and water control are designated by the acronyms C, LSW, D, BND and W, respectively. Bars under different letters are significantly different with  $p < 0.05$ .



**Origin of the chemical alarm cues**

**Chapter 3. Response to chemical alarm cues under weakly acidic conditions: a graded loss of antipredator behaviour in juvenile rainbow trout (*Oncorhynchus mykiss*).**\*

**Introduction**

Despite recent reductions in sulphur emissions, acid precipitation remains a serious and persistent environmental problem (Schindler 1988; Stoddard et al. 1999; Clair et al. 2001). Acidification can cause increased physiological stress in affected organisms (Lacroix et al. 1985; Baker et al. 1996), reduce survival (reviewed in Doka et al. 1997), alter behaviour (Gunn 1986; Åtland 1998) and reduce species richness and/or abundance in an ecosystem (Baker et al. 1996; Gerold et al. 2000). Much of this work has focussed on the effects of intermediate to heavily acidified conditions (pH < 5.5; Gunn 1986, Peterson et al. 1989) and far less attention has been directed toward investigating the impacts of weak (sub-lethal) acidification (but see Kitamura and Ituka 2000; Leduc et al. 2003, 2004a). Many regions of Eastern Canada and the American Northeast can be characterized as weakly acidic (pH 5.5 - 6.5; Clair et al. 2001; Holt et al. 2003). Fluctuations between circum-neutral and weakly acidic conditions may occur as a function of daily or seasonal variations in anthropogenic acid inputs (Baker et al. 1996; Komai et al. 2002) that may exacerbate naturally acidic sources (Dangles et al. 2004). As such, the environmental impacts of weakly acidic conditions can be persistent and may have significant impacts on aquatic fauna (Kitamura and Ituka 2000; Leduc 2004a, 2006, 2007b).

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\* Leduc, A.O.H.C., Lamaze, F.C., McGraw, L. & Brown, G.E. (in press). *Water, Air, and Soil Pollution*.

Recently, research has examined the potential effects of weakly acidic conditions (pH ~ 6.0) on the chemosensory assessment of local predation risk by several prey fish species (Brown et al. 2002, Leduc et al. 2003, 2004a, 2006). Such chemosensory risk assessment is performed by wide-ranging and taxonomically diverse freshwater fishes, including salmonids (Brown and Smith 1997; Leduc et al. 2006) that may rely on damage-released chemical alarm cues (reviewed in Chivers and Smith 1998) to assess predation threats. These chemical cues are released following mechanical damage to the skin (Chivers and Smith 1998; Brown 2003) that likely occurs during a predation event and, when detected by nearby conspecifics and sympatric heterospecifics, can elicit dramatic short-term increases in species typical antipredator behaviour. Recent laboratory and semi-natural field enclosure studies have demonstrated that responding to these alarm cues increases the probability of survival during staged-encounters with live predators (Mirza and Chivers 2001b, 2003). As such, the ability to detect and respond to these cues likely contributes significantly to individual fitness, population recruitment and growth.

Recent laboratory and field studies have demonstrated that relatively minor changes in ambient acidity result in a significant impairment in the ability of prey fishes to detect and respond to these critically important information sources (Brown et al. 2002; Leduc et al. 2003, 2004a, 2006). In these studies, exposures to chemical cues did not elicit a significant increase in alarm behaviour under weakly acidic conditions (pH 6.0) while under neutral conditions, normal alarm responses (e.g., reduction in activity, increased shelter use and increased group cohesion) occurred. In these studies, however, chemosensory assessment was verified under two relatively distinct levels of ambient

acidity: circum-neutral (pH ~ 7.0) or weakly acidic (pH ~ 6.0). Graded acidification may occur under natural conditions following increasing acid inputs (Van Sickle et al. 1996; Wigington et al. 1996). It remains unknown whether the antipredator response to chemical alarm cues likewise follows a graded function, decreasing in intensity with increasing ambient acidity or if, alternatively, an acidity threshold exists under which no alarm response occurs. This may be of critical importance for fishery management, given that the concentration of chemical alarm cues should be directly related to the proximity of a predation event. Under normal (neutral) conditions, prey fishes should be able to assess local predation risk based on the concentration of alarm cue detected (Lawrence and Smith 1989; Dupuch et al. 2004) while under weakly acidic conditions, chemosensory risk assessment abilities may either be reduced or absent. Furthermore, relating the intensity of an alarm response to chemical cues under graded pH conditions may indirectly inform about the potential degradation mechanism of these chemical alarm cues (Brown et al. 2002). For instance, are the chemical cues degraded proportionally to ambient acidity or does an absolute threshold exist for an ‘all or nothing’ response (Brown et al. 2006)?

I therefore conducted this series of laboratory studies to test the effects of a range of pHs on the detection and response to conspecific chemical alarm cues to determine: 1) the threshold pH at which the antipredator response is lost and 2) if the loss of response is graded or if an absolute threshold exists. To determine at which pH level the alarm response to chemical cues is lost, I exposed juvenile rainbow trout (*Oncorhynchus mykiss*) to conspecific alarm cues or controls of distilled water that had been left untreated (i.e., not buffered) or buffered to pH of 6.6, 6.4, 6.2 or 6.0 with the addition of



minute amounts of H<sub>2</sub>SO<sub>4</sub>. To test whether the loss of chemosensory function is graded or if a threshold exists, I used a repeated measures design in which I exposed juvenile trout to alarm cues at varying pH or distilled water.

## **Material and methods**

### EXPERIMENT 1

#### ***Test fish***

Juvenile rainbow trout were obtained from Pisciculture des Arpents Verts, Ste. Edwidge de Clifton, Québec. Trout were held in 100 L circular flow-through holding tanks and supplied with continuously filtered water at approximately 500 mL/minute. Temperature was approximately 14°C (range: 11 to 16°C). Prior to experiments, trout were fed, *ad-libitum*, twice daily with commercial trout chow and held on a 12:12 light:dark cycle.

#### ***Test tanks***

Test tanks consisted of a series of 37 L glass aquaria, filled with 35 L of dechlorinated tap water (16°C, pH ~ 6.9) and a gravel substrate. Water in the test tanks was not filtered. Along the back wall of each tank I attached a single air stone to which I attached an additional 2.5 m length of airline tubing to allow for the injection of test cues without disturbing the test fish. Three sides of the tanks were covered to ensure visual isolation between test tanks. In addition, I marked a horizontal line on the front and back walls of the tanks at 9 cm from the substrate (i.e., one third of the height) to facilitate recording time near the substrate (see below). Tanks were drained and thoroughly rinsed with tap water between each trial.

### ***Stimulus preparation***

I generated my stock alarm cues from 26 juvenile rainbow trout (mean  $\pm$  SD standard length =  $24.7 \pm 2.4$  mm). Donor trout were killed with a blow to the head (in accordance with Concordia University Animal Care Committee protocol #AC-2005-BROW). I removed a skin fillet from either side of the donor and immediately placed these into 100 mL of chilled, glass-distilled water. Skin fillets were then homogenized and filtered through polyester filter floss to remove any remaining tissue particles. I collected a total of  $27.77 \text{ cm}^2$  of skin and diluted this to a final volume of 315.30 mL. Thus, the final concentration of alarm cue was similar to that used in previous studies (Brown and Smith 1997; Leduc et al. 2004a). Alarm cues were frozen in 20 mL aliquots at  $-20^\circ\text{C}$  until needed. As a control, I likewise froze 20 mL aliquots of distilled water.

I prepared a stock acid solution by initially diluting 1 mL of 95%  $\text{H}_2\text{SO}_4$  into 1000 mL of distilled water. I further diluted this solution (1:100) in distilled water and used this final  $\text{H}_2\text{SO}_4$  solution for adjusting the pH of the alarm cues and the distilled water (control) stimuli. The use of the dilute  $\text{H}_2\text{SO}_4$  allowed for accurate control of the final pH of the stimuli.

### ***Experimental protocol***

I placed individual trout into test tanks and allowed them a 24-hour acclimation period before testing. Trials were divided into a 5-minute pre-stimulus and a 5-minute post-stimulus injection observation period. Individual test tanks were randomly assigned to either the control (distilled water) or experimental (alarm cues) treatments. Prior to the pre-stimulus observation period, I withdrew and discarded 60 mL of tank water from the

stimulus injection tube (to remove any stagnant water) and withdrew and retained an additional 60 mL of water. Immediately following the prestimulus observation, I injected 5 mL of alarm cue or distilled water. For both experimental and control stimuli, I either left them untreated (pH ~ 6.9) or adjusted them to pH 6.6, 6.4, 6.2, or 6.0 with the addition of H<sub>2</sub>SO<sub>4</sub> (volume ranging between 0.03-0.05 mL). The order of treatments (pH and stimulus type) was randomized. I conducted a total of 10 replicates for each treatment combination. Mean ( $\pm$  SD) standard length of trout at time of testing was 24.69  $\pm$  2.44 mm.

During both pre- and post-stimulus observation periods, I recorded: 1) number of foraging attempts, 2) time spent moving and 3) time spent in the bottom third of the test tank (i.e., close the substrate).

### ***Statistical analysis***

I calculated the change in each behavioural measure (post-stimulus – pre-stimulus) for both control and experimental trials and used these difference scores as dependent variables in all analyses. Since the behavioural measures are highly correlated, I tested for the overall effects of alarm cue and pH using a MANOVA. Due to the presence of a significant chemical alarm cue x pH interaction (see below), I subsequently conducted individual MANOVAs for each pH level.

## **EXPERIMENT 2**

The results of Experiment 1 (see below) suggest that the response to conspecific alarm cues were not completely lost at pH ~ 6.4. Therefore, I conducted the second experiment

to allow me to further explore this possibility by exposing individual trout to a range of acidified alarm cues (or a control of distilled water) using a repeated measures design.

### ***Test fish***

Same as in Experiment 1 (see above).

### ***Test tanks***

Same as in Experiment 1 (see above).

### ***Stimulus preparation***

I collected alarm cues from juvenile rainbow trout as described above. I collected skin extracts from a total of 34 juvenile trout (standard length =  $44.47 \pm 0.85$  mm) donors.

The final concentration for trout skin extract was similar to that described above (103.65  $\mu\text{g cm}^{-2}$  in 1175 mL). As above, alarm cues and the controls of distilled water were frozen in 20 mL aliquots until needed.

### ***Experimental protocol***

Using a repeated measures design, I exposed individual trout ( $n = 20$ ) to untreated alarm cue (pH  $\sim 6.9$ ), and alarm cue buffered to pH 6.6, 6.4, 6.2 (as described above) and a control of distilled water. The protocol and behavioural measures recorded were as in Experiment 1. The order of treatments was randomized. Following the post-stimulus observation period, individual trout were moved to an identical test tank, and allowed 24

hours before repeated testing until they had been exposed to all treatments. Mean ( $\pm$  SD) standard length of trout at time of testing was  $45.12 \pm 1.09$  mm.

### ***Statistical analysis***

I used repeated measures MANOVAs to examine the overall effect of alarm cue pH on the antipredator behaviour of trout exposed to conspecific alarm cues. In order to test the hypothesis that the loss of response is graded (i.e. linear versus quadratic), I conducted planned contrast analyses for the behavioural measures which yielded significant univariate repeated measures effects. If the effect of reduced pH is graded, I predict a linear relationship between acidification and response intensity.

## **Results**

### **EXPERIMENT 1**

The overall MANOVA revealed significant effects of both stimulus type (alarm cue versus distilled water) and pH (Table 3.1). Moreover, I found a significant interaction between these main effects (Table 3.1; Figure 3.1). Subsequent comparisons revealed no effect of stimulus type when the stimuli were buffered to pH 6.4 and below (Table 3.1; Figure 3.1) but there was a significant difference between alarm cue and distilled water at pH 6.6 and the untreated conditions (Table 3.1; Figure 3.1). However, there are clear trend evidences of a partial response at pH 6.4. In fact, posthoc t-tests revealed significant differences between alarm cue (pH 6.4) and distilled water (pH 6.4) for the change in number of foraging attempts ( $t_{16} = 2.12$ ,  $p = 0.024$ ; Figure 3.1) and time spent moving ( $t_{16} = 1.92$ ,  $p = 0.035$ ; Figure 3.1). There was no significant difference between

alarm cues and distilled water at pH 6.4 for change in time on the substrate ( $t_{16} = 0.21$ ,  $p = 0.822$ ; Figure 3.1). This shows that the alarm response of trout weakens at a pH of 6.4 to disappear completely at pH 6.2.

## EXPERIMENT 2

The repeated-measures MANOVAs revealed significant overall effects of pH on the intensity of the alarm response of rainbow trout (Table 3.2). Subsequent repeated measures ANOVAs revealed that these overall effects were due to significant differences in the change in foraging rate and time spent moving (Table 3.2; Figure 3.2). I found no significant repeated measures effects for the change in time on substrate (Table 3.2).

To test for a graded decline in alarm cue function associated with reduced pH, I conducted planned contrast analyses. These results demonstrate that in response to conspecific alarm cues, the loss of function due to reduced pH followed linear trend for both foraging (linear contrast:  $F_{1,19} = 10.05$ ,  $p = 0.005$ ; quadratic contrast:  $F_{1,19} = 0.026$ ,  $p = 0.88$ , Figure 3.2) and time spent swimming ( $F_{1,19} = 20.61$ ,  $p < 0.001$ ; quadratic contrast:  $F_{1,19} = 0.12$ ,  $p = 0.73$ , Figure 3.2). Planned contrasts were not conducted for the change in time on substrate as I found no significant repeated measures effect (Table 3.2).

## Discussion

These results demonstrate that the loss of response towards damage-released chemical alarm cues by juvenile rainbow trout is graded and alarm cues cease to be detectable (i.e., fail to elicit an increase in antipredator behaviour) below pH 6.4. The results of the first experiment show that at pH 6.4, trout exhibited only a weak response to alarm cues and

no response occurred at pH 6.2 and below. The results of the second experiment suggest that the loss of response is graded (i.e., the response intensity decreases with increasing acidity) hinting that at a pH of 6.4, the degradation of the alarm cue is not complete. Combined, these results suggest that even minor fluctuations in ambient pH can impair the ability of juvenile salmonids to respond to conspecific alarm cues. Given the demonstrated importance of alarm cues in the assessment of local predation threats, this impairment may exert a significant sub-lethal effect on salmonid populations.

Previous works have demonstrated chemosensory impairment to various chemicals under acidified conditions. Moore (1994) showed significantly reduced electrophysiological responses to sex pheromones at pH 6.5, 5.5 and 4.5 in male Atlantic salmon (*Salmo salar*). At pHs ranging from 5.5 to 6.5, significantly higher concentrations of sex pheromones (testosterone and ovulated female urine) were necessary to trigger an electrophysiological response of similar intensity to that under neutral conditions (Moore 1994). Similarly, Hara (1976) and Thommesen (1983) showed that the response of rainbow trout to different amino acids was also highly pH dependent (cited from Moore 1994). Although weak acidification may reduce the ability to detect various chemicals, the mechanisms involved in the detection loss may vary. For instance, Moore (1994) suggested that acidification results in lower detection abilities of affected organisms and after a recovery time, the electrophysiological sensitivity to the pheromones returned to pre-acidic levels. In this experiment, I only acidified the alarm cues with a minute amount of acid (approximately 0.04 mL of acid) thus the final tank pH between each treatment did not vary significantly and cannot explain the observed loss of response. Brown et al. (2002) conducted a sequential exposures experiment to

chemical alarm cues in which two neutral treatments were separated by a weakly acidic treatment (at 48 hours intervals). The results of this experiment showed observable alarm responses in two cyprinid species under both neutral treatments while no response occurred under the intermediate weakly acidic treatment. These authors concluded that the chemical cues were somehow degraded (rendered non-functional) from a chemical change to alarm cue molecule and that no permanent olfactory receptor damage occurred (Brown et al. 2000, 2002). Likewise, Leduc et al. (2004b) showed that paired exposures of alarm cues and a novel odour elicited significant increases in alarm behaviour. For instance, in their experiment individuals were able to recognize acidified and neutral novel odour as long as it was paired with alarm cues that were not acidified. This showed that the pH of the novel odour had no effect on the alarm response suggesting that the olfactory function of rainbow trout at pH ~ 6.0 was not significantly affected. Taken together, these results allow me to conclude that chemosensory functions in freshwater fishes can be affected by weak changes in ambient pH from different impairment mechanisms, while no permanent chemosensory loss appears to occur.

The observed graded loss of function of alarm cues may occur from two non-mutually exclusive mechanisms. Firstly, the observed loss of response may be due to a concentration effect in which the functional concentration of the alarm cues is reduced from a degradation effect (Brown et al. 2002). Several authors have demonstrated that the intensity of the antipredator response in a number of prey fishes is proportional to the concentration of alarm cues detected (Brown et al. 2001b; Mirza and Chivers 2003; Brown et al. 2006). However, this mechanism is unlikely to account for the observed response patterns as Mirza and Chivers (2003) demonstrated a non-graded response by



juvenile rainbow trout to decreasing concentrations of conspecific alarm cues. Alternatively, the chemical structure of the alarm cues may change with increasing acidity, reducing its binding affinity with the olfactory receptors of trout. Kelly et al. (2006) have shown that changing the ratio of purine skeletons while holding the absolute concentration of nitrogen-oxides (the active component of the Ostariophysan alarm cue; Brown et al. 2000) constant resulted in a graded response pattern in fathead minnows (*Pimephales promelas*). Thus, it is possible that subtle changes in the trout alarm cues due to ambient acidity might reduce its overall 'detectability', resulting in a graded loss of response. Although I cannot ascertain the exact chemical mechanism responsible for the observed loss in alarm function, the results clearly demonstrate that even relatively slight changes in ambient acidity have considerable impacts on chemosensory risk assessment in juvenile salmonids. In both graded and non-graded loss of alarm response, no loss in olfactory functions of the test fish likely occurred (Brown et al. 2002; Leduc et al. 2004a).

The threat-sensitive predator-avoidance hypothesis predicts that as the concentration of chemical alarm cues decreases, the intensity of the antipredator response will likewise decrease (Helfman 1989; Brown et al. 2006). Eventually there should be a point where no overt antipredator behaviour can be observed (i.e., there is a behavioural response threshold). As such, if the degradation of the alarm cues under intermediate pH conditions is only partial, the alarm cues may still convey ecologically relevant information about local danger, even in the absence of an observable response (Brown et al. 2001a; Mirza and Chivers 2003). Indeed, prey fish exposed to concentrations of alarm cues insufficient to trigger an observable alarm response still survived predator

encounters better than 'naïve' fish (Mirza and Chivers 2003). As such, weak acidification may still allow for partial chemosensory risk assessment. In this experiment, I suggest that a graded decrease in alarm response occurs from neutral (pH ~ 7.0) to an approximate pH of 6.4. Under this apparent threshold, the chemosensory alarm function of rainbow trout is impaired (non-functional). It is unknown whether prey fish suffer increased predation costs under weakly acidic conditions. A clear next step will be to investigate this question.

Prey fishes, including salmonids, are likely exposed to a varying range of pH, depending upon seasonal changes or daily rainfall (Gunn 1986; Baker et al. 1996; Wigington et al. 1996). This is an important issue since ambient pH may vary with the specific buffering capacities of microhabitats (Doka et al. 2003). As such, episodic acidification events may lead to rapid change in ambient pH during which prey fishes may not be able to rely on chemical alarm cues to assess ambient risks. The presence of chemosensory information may therefore be variable in fluctuating pH conditions. Given the demonstrated importance of chemosensory risk assessment cues, the loss of alarm cue response, even if temporary, may represent a significant sub-lethal effect of anthropogenic acidification of natural waterways.

Table 3.1. Overall MANOVA significance values between both stimulus type (alarm cues versus distilled water) and pH on trout change in antipredator predator.

	F	df	p
MANOVA			
Stimulus	5.83	3, 88	= 0.001
pH	4.00	4, 90	= 0.008
Stimulus x pH	3.68	4, 90	= 0.008
Untreated	3.56	3, 16	= 0.037
pH 6.6	3.41	3, 16	= 0.043
pH 6.4	2.09	3, 16	= 0.14
pH 6.2	0.23	3, 16	= 0.88
pH 6.0	0.56	3, 16	= 0.65

Significance was established at  $p \leq 0.05$ .

Table 3.2. Results of repeated-measures MANOVAs and ANOVAs (MANOVAR and ANOVAR, respectively) on the intensity of the alarm response of rainbow trout at different pH.

	F	df	p
<b>MANOVAR</b>	8.15	4, 76	< 0.001
<b>ANOVAR</b>			
Foraging	5.59	4, 16	= 0.005
Moving	4.56	4, 16	= 0.012
Time on Substrate	1.23	4, 16	= 0.37

Significance was established at  $p \leq 0.05$ .

Figure 3.1. Mean ( $\pm$  standard error) differences (post - pre) in: a) number of feeding attempts, b) time in seconds spent in motion and c) time in seconds spent motionless on the substrate for rainbow trout (*Oncorhynchus mykiss*) exposed to the alarm cues under different acidic treatments. Dark bars correspond to alarm cue treatments whereas open bar correspond to water (control) treatments.

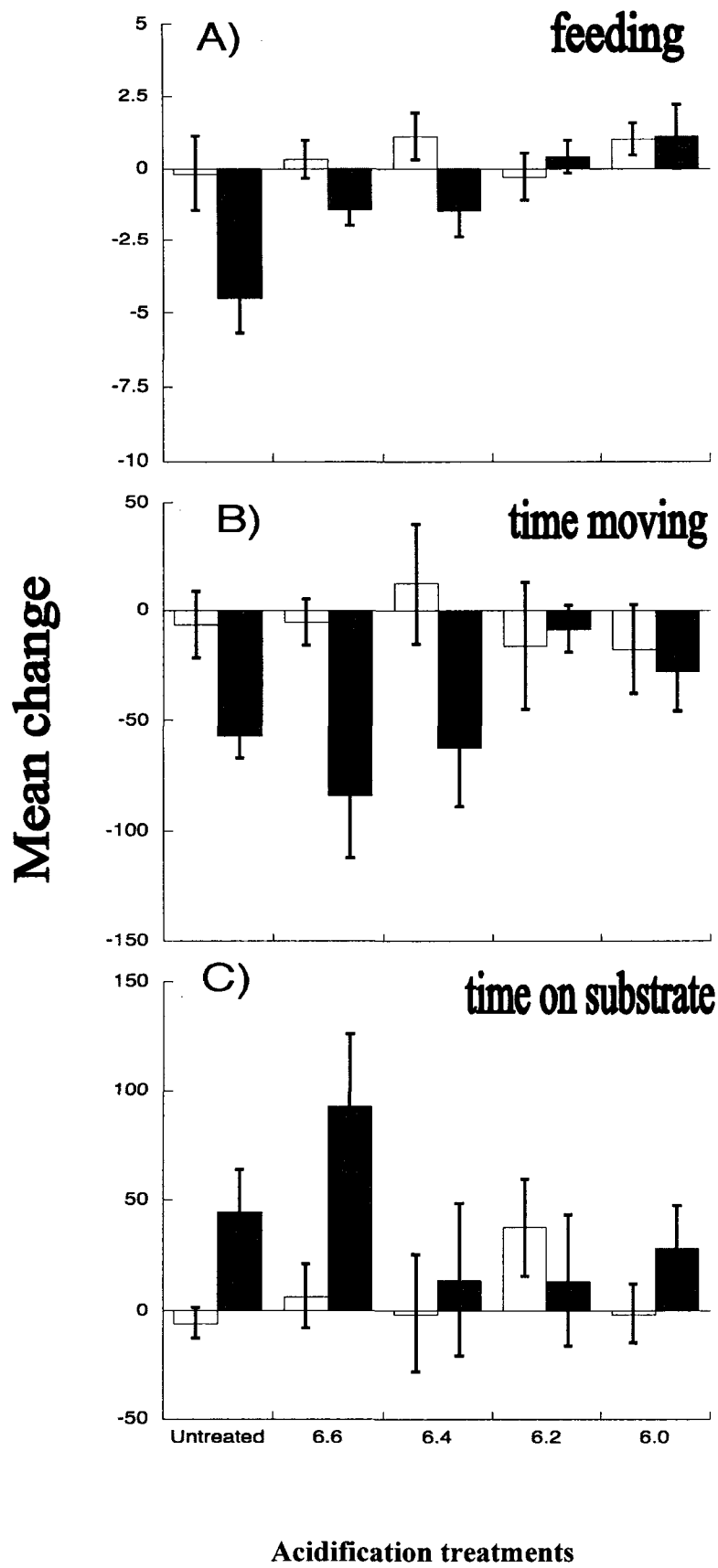
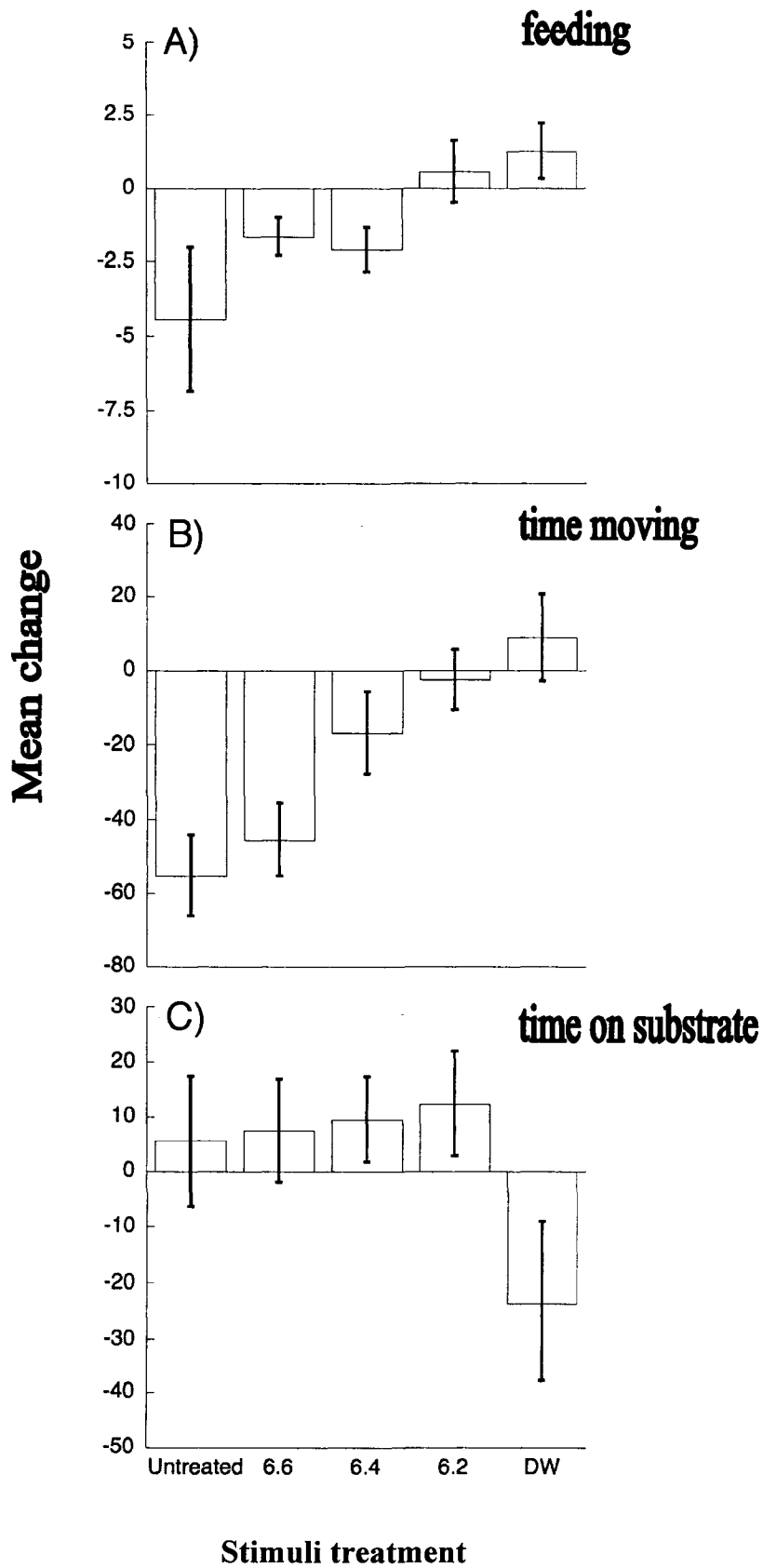


Figure 3.2. Mean ( $\pm$  standard error) differences (post - pre) in: a) number of feeding attempts, b) time in seconds spent in motion and c) time in seconds spent motionless on the substrate for rainbow trout (*Oncorhynchus mykiss*) exposed to the alarm cues in a repeated measures treatment.





## **Chapter 4. Effects of ambient acidity on chemosensory learning: example of an environmental constraint on acquired predator recognition in wild juvenile Atlantic salmon (*Salmo salar*).\***

### **Introduction**

The ability to detect, avoid and escape from predators is of prime importance for the survival of prey individuals (Sih 1987; Lima and Dill 1990). Ineffective predator recognition and subsequent attack or capture by predators reduces (or eliminates) further reproductive efforts. In spite of this, prey individuals must balance the conflicting demands of fitness enhancing activities, for instance feeding and mating, against predator avoidance. As such, a strong selection gradient should favour the ability to differentiate between dangerous and non-dangerous stimuli. While such an ability to recognize potential threats may be innate (Brown and Chivers 2005), ample empirical evidence of learned recognition of predators exists in the literature (Chivers and Smith 1994a, b; Griffin et al. 2001; Brown 2003). For example, learned predator recognition has been documented in invertebrates (Rochette et al. 1998; Wisenden et al. 1999; Wisenden and Millard 2001), amphibians (Kiesecker et al. 1996; Woody and Mathis 1998), birds (Curio et al. 1978; McLean et al. 1999), mammals (Griffin et al. 2001, Griffin and Evans 2003) and fish (Mirza and Chivers 2000), and may occur with biologically relevant and irrelevant stimuli (Yunker et al. 1999). Accordingly, it appears that this phenomenon is widespread.

Within aquatic ecosystems, chemically mediated predator recognition learning

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\* Leduc, A.O.H.C., Roh, E., Breau, C., Brown, G.E. (2007). *Ecology of Freshwater Fishes*, **16**, 385-394.

has been well documented (Brown and Chivers 2005). Such learning can be mediated via damage-released chemical alarm cues (Smith 1992, 1999). These chemical cues have been studied extensively in both invertebrates (Rochette et al. 1998; Wisenden et al. 1999; Wisenden and Millard 2001) and vertebrates (Chivers and Smith 1998; Brown 2003). In fish, chemical alarm cues are typically passively released from the injured skin of prey following a predation event (Smith 1992, 1999). Their detection by nearby conspecifics often triggers an increase in innate species-typical antipredator behaviour (Chivers and Smith 1998). Given the context of their release and the behavioural responses they trigger, these chemical cues can facilitate the association of an originally neutral stimulus (such as a novel predator odour) with a potential predation threat (Brown and Chivers 2005). Recent studies have demonstrated that chemically mediated learned predator recognition leads to greater survival benefits during subsequent predator encounters (Mirza and Chivers 2000, 2001b; Darwish et al. 2005).

Although damage-released chemical alarm cues may give valuable information regarding local risk, their function may be limited under acidic conditions. Both laboratory (Brown et al. 2002; Leduc et al. 2003, 2004a) and field studies (Leduc et al. 2004a, 2006) have shown that the ability to detect and respond to conspecific and heterospecific alarm cues is impaired under relatively weak acidic conditions (pH ~ 6.0). Likewise, chemosensory-mediated learning from damage-released alarm cues may also be hindered under acidic conditions. For example, Leduc et al. (2004b) demonstrated in a laboratory experiment that hatchery-reared rainbow trout exposed to the novel odour of a yellow perch (*Perca flavescens*) paired with conspecific chemical alarm cues at a pH

of 7.0 learned to recognize the novel odour as a potential threat. However, when exposed to acidified alarm cues (to a pH of 6.0), trout failed to recognize the novel odour.

Laboratory conditions, however, often lack the ecological realism of field studies and as such, many researchers have urged for field verifications of laboratory results (Magurran et al. 1996; Smith 1997; Wisenden et al. 2004). Laboratory conditions may represent a suite of stressful disturbances, unrepresentative of the ecological conditions that individuals face in the wild (Magurran et al. 1996). In addition, unlike wild fish, hatchery-reared fish used in laboratory experiments likely lack previous experience with predation risks, and may differ in their ability to learn novel predator cues (Berejikian 1995; Álvarez and Nicieza 2003). Of particular importance, acidification found in natural environments may not be exclusively from a single source (e.g. sulfuric acid). Multiple acidification sources, both natural and anthropogenic may contribute to the reduction of ambient pH (Guerold et al. 2000; Dangles et al. 2004) and may translate into different effects on the chemical alarm cues. Such potential differences in alarm cue degradation between laboratory and natural conditions may influence chemosensory learning of a novel stimulus under acidic conditions. For instance, failure to measure a response to chemical alarm cues in natural conditions (Leduc et al. 2004a) does not imply that prey individuals are not gaining information about prevalent predation risks provided by the chemical cues. Concentrations of alarm cue that fail to trigger an overt (observable) alarm response may still facilitate the acquisition of information about local predation risks (Brown and Smith 1996; Brown et al. 2001a, b) and such sub-threshold concentrations can still translate into better survival for conditioned prey fish (Mirza and Chivers 2003). Consequently, in natural settings, it is unknown whether the measured

impairment of chemical alarm cue detection found under acidic conditions (Leduc et al. 2004a) translates into impaired chemosensory learning of a novel cue, thus imposing an environmental constraint on this learning mechanism.

In this experiment, I assessed, under fully natural conditions, whether the ambient acidity had any effects on the ability of wild juvenile Atlantic salmon (*Salmo salar*) to recognize a novel stimulus (odour) after one paired exposure with conspecific chemical alarm cues. To investigate this question, I conducted observation trials in two nursery streams, that varied in acidity (see below), used by wild Atlantic salmon. I predicted that if juvenile salmon were able to detect conspecific chemical alarm cues (even in the absence of an observable response), they would associate a novel lemon essence odour (see below) as an indication of danger. As such, 'conditioned' fish should exhibit typical alarm responses following exposure to the novel odour alone. However, if no chemosensory alarm function is available under acidic conditions (e.g., if the chemical alarm cues are not detectable), I predicted that no learning would occur and that no alarm response would be detectable following exposure to the novel odour.

## **Materials and methods**

### ***Study sites***

I conducted this experiment in Northumberland County in New Brunswick, Canada, using two third order tributaries of the Little Southwest Miramichi River (Figure 4.1) having different mean acidity levels. Catamaran Brook and Devil's Brook are both nursery streams used by wild Atlantic salmon. During this experiment, Catamaran Brook was considered neutral (mean  $\pm$  SE pH = 7.14  $\pm$  0.09) whereas Devil's Brook was

considered acidic (mean  $\pm$  SE pH = 6.08  $\pm$  0.07). In each of these streams, I chose a section of approximately 100 m that were similar in terms of width, depth, current speed, canopy cover and substratum complexity (see below; Table 4.1).

### ***Test subjects***

I used wild juvenile Atlantic salmon to test for their ability to acquire the recognition of a novel odour from a single pairing with conspecific chemical alarm cues and to assess whether ambient acidity had any effect on this learning mechanism. Smith (1999) reported that a single exposure to the alarm cue is sufficient to elicit an alarm response. I chose Atlantic salmon to investigate this question as they possess a suite of distinctive advantages to conduct such a field study. Firstly, given their territoriality and site fidelity behaviour (Steingrímsson and Grant 2003), Atlantic salmon are easy to follow on a day-to-day basis, allowing for easy visual observation in their habitat. Secondly, several studies have demonstrated that conspecific chemical alarm cues elicit observable alarm responses in a number of salmonid species including Atlantic salmon (Brown and Smith 1997; Mirza and Chivers 2001a; Leduc et al. 2004a). Third, as Atlantic salmon avoid pHs below *c.* 4.5 (Gunn and Noakes 1986; Peterson et al. 1988; Åltand 1998), the conditions found in the test streams (minimum pH  $\sim$  6.01) should not have created severe physiological stress that would significantly affect their ‘normal’ behaviour. Finally, given their social and commercial interests and the decline in the number of wild Atlantic salmon, I hope this study may shed light on one reason as to explain the stock’s inability to recover from a depleted state.

### ***Stimuli production***

To conduct this experiment, I used either a juvenile Atlantic salmon chemical alarm cue or stream water (experimental vs control) paired with lemon essence (Pure Lemon Extract, McCormick Canada, London, Canada) as a novel odour. I chose lemon essence as a novel odour for two main reasons. Firstly, given its origin, it is extremely unlikely that the juvenile salmon had any prior exposure to this odour, thus allowing us to test a truly novel stimulus. Yunker et al. (1999) have demonstrated that association of danger can occur with biologically irrelevant stimuli. Secondly, lemon essence is clear, water-soluble and dilutes well in water thus having suitable physical properties for this study.

On June 17<sup>th</sup> 2004, I captured 14 juvenile Atlantic salmon (mean  $\pm$  SD fork length =  $4.58 \pm 0.23$ ) from the Little Southwest Miramichi River to be used as skin donors from which the chemical alarm cue was generated. After donor fish were humanely killed (in accordance to Concordia Animal Care Committee Protocol AC-2005-BROW), I removed a skin fillet from each side of their bodies. I collected a total of 44.9 cm<sup>2</sup> of skin that I homogenized in an ice-chilled container filled with 530 mL of stream water at a pH of 7.07. This final concentration is known to elicit an alarm response in salmonids (Brown and Smith 1997; Mirza and Chivers 2001a; Leduc et al. 2004a). To remove any particulate matter, I filtered the solution through poly-filter wool fiber. As a control, I used stream water collected prior to the start of each trial (see below). As a novel odour, I used lemon essence (see above) that I diluted into unchlorinated well water (1: 43) until only a faint odour was detectable. The resulting novel odour dilution had a pH of 7.14. I prepared 20 mL aliquots of each alarm cue and novel odour and froze them at  $-20^{\circ}\text{C}$  until needed.

### *Experimental protocol*

This experiment was conducted in two phases. In both Catamaran Brook and Devil's Brook, I initially conditioned juvenile salmon with a cocktail of either chemical alarm cues or stream water (for the experimental or control stimuli, respectively) paired with the lemon odour. Twenty-four hours later, I tested the same (conditioned) fish with the lemon odour alone to assess if they responded with an alarm response.

For both the conditioning and the testing phases, I used individually tagged wild YOY Atlantic salmon. Using dip nets while snorkelling, I haphazardly captured salmon in the chosen sections of Catamaran Brook and Devil's Brook. On August 8<sup>th</sup> and August 18<sup>th</sup>, I captured and tagged 45 individual salmon in Catamaran Brook (mean  $\pm$  SD fork length (cm) =  $4.71 \pm 0.38$ ) and 47 in Devil's Brook (mean  $\pm$  SD fork length (cm) =  $4.78 \pm 0.33$ ) respectively. Each captured subject was tagged by implanting minute amounts of red, green, pink, orange or blue elastomer pigments in the epidermis (for a fully detailed protocol, see Dewey and Zigler 1996; Steingrímsson and Grant 2003). This tagging technique is believed to not significantly affect individuals' behaviour and allows for the tracking of known individuals during the course of the study (Steingrímsson and Grant 2003). Once tagged, each fish was released at the location of its capture.

### *Phase one: conditioning*

The conditioning phase took place on August 12<sup>th</sup>, 14<sup>th</sup> and 16<sup>th</sup> in Catamaran Brook and on August 20<sup>th</sup>, 22<sup>nd</sup> and 24<sup>th</sup> in Devil's Brook. After I found a tagged salmon while snorkeling in the test sites, I positioned myself approximately 1.5 m upstream from the test fish at an angle of approximately 45 degrees relative to the water current. I used this

positioning to reduce the formation of artificial turbulence that may have interfered with the test fish. To let the test fish acclimate to my presence, I waited a minimum of two minutes before starting a behavioural trial (see below). A trial did not start unless the fish was behaving 'normally' (i.e., that it was feeding and moving). Trials consisted of 5 minutes of pre- and 5 minutes of post-stimulus injection observation. After the first 5 minutes elapsed, I injected one of the two stimuli (randomly chosen) paired with the novel lemon odour using two 60 mL syringes attached together designated for the appropriate stimulus and the novel odour. All trials were video-recorded using an underwater camera (Sea View™) positioned approximately 1.5 m from the test fish. From this distance, detailed behavioural observations could be made with accuracy (Leduc et al. 2004a; Leduc et al. 2006). In total, 40 and 39 salmon were conditioned in Catamaran Brook and Devil's Brook respectively.

#### *Phase two: testing*

Between 24 and 27 hours after individuals were conditioned, I conducted the testing trials (on August 13<sup>th</sup>, 15<sup>th</sup> and 17<sup>th</sup> in Catamaran Brook and on August 21<sup>st</sup>, 23<sup>rd</sup> and 25<sup>th</sup> in Devil's Brook). This was done to assess if the test subjects would show an alarm response after being exposed to the novel odour alone. I used the same protocol as in the conditioning phase except that I exposed the conditioned fish to the novel lemon odour alone (i.e., not paired with either chemical alarm cue or stream water). All conditioned salmon were tested during the testing phase (40 and 39 in Catamaran Brook and Devil's Brook respectively).



To assess whether the environmental conditions in which the fish were tested potentially had any influence on their response, I measured depth, current speed, percentage of canopy cover, pH, dissolved oxygen, substrate complexity, percentage of cloud cover and water temperature (Table 4.1). The current speed was recorded from 5 cm below the water surface using a Flo-Mate velocity meter (Marsh McBirney Inc., Frederick, MD). pH, dissolved oxygen, and water temperature, were measured using a WTW-P 4 MultiLine meter (WTW, Weilheim, Germany), which was calibrated with standard solutions (pH: technical buffers pH 4.01 and 7.01; conductivity: conductivity cells 0.01 mol/l KCl; dissolved oxygen: alkaline electrolyte solution) at the start of each day. The substrate complexity was determined by using a one-meter long flexible metal wire that I moulded to the substrate at the focal location of each test subject (i.e., the location where they received the stimuli). A value was obtained by measuring the linear distance between the ends of the wire after it was molded to the substrate. Since the metal wire had a length of 1 meter, a substrate complexity value of 1 would indicate that the substrate is flat (i.e., no complexity) while a lower number would indicate greater complexity. I averaged environmental values (physical and chemical) obtained in the conditioning and testing phases for each test site and compared these values across the two study streams using an ANOVA (Table 4.1) to assess if they differ significantly.

### ***Behavioural measures and statistical analysis***

To determine whether individuals learned to recognize the stimuli as a potential threat, I measured three behavioural parameters typical of an alarm response. I quantified the number of feeding attempts, the time (in seconds) spent in motion and the time (in

seconds) spent motionless on the substrate. I defined a feeding attempt as a change of location of at least half a body length in which a pecking motion occurred (either in the water column or on the substrate). The time in motion was measured when an individual changed its location by at least one body length, whereas the time motionless on the substrate was measured when an individual stayed in contact with the substrate without changing its location. All video-recorded trials were analyzed by the same observer on a 14-inch (35 cm) Toshiba™ flat-screen monitor without prior knowledge of the experimental treatment or stimulus type.

For each behavioural measure, I determined the occurrence of an alarm response by calculating differences in behavioural scores between the pre- stimulus and the post-stimulus period (post-stimulus minus pre-stimulus) scores. A decrease in feeding attempts and/or in time spent moving and/or an increase in time spent motionless on the substrate was interpreted as an alarm response (Chivers and Smith 1998; Leduc et al. 2004a, 2006).

I used a repeated measures MANOVA to test for any overall effects of acidity (neutral versus acidic) and stimulus type (salmon alarm cues or stream water) on the intensity of the alarm response parameters measured using streams (i.e., level of acidity) and stimulus type as independent variables. I used the multivariate approach, since the three behavioural measures are likely highly correlated; for example, a reduction in time moving would decrease frequency of foraging attempts. Using subsequent repeated measures MANOVA, I compared the overall effect of stimulus type on the intensity of the alarm response during the conditioning and the recognition phases separately for each stream.

To control for differences associated with the test fish populations, I compared the baseline (pre-stimulus) values of each behaviour between the two studied streams using an ANOVA. SPSS 11 was used to conduct all statistical analysis.

## **Results**

The initial repeated measures (RM) MANOVA (R square = 0.34) revealed a significant overall effect of stimulus type (alarm cues versus stream water;  $F_{3, 73} = 12.95, p = 0.001$ ) and stream (neutral versus acidic;  $F_{3, 73} = 7.01, p = 0.006$ ) on the antipredator response of juvenile salmon. More importantly however, I found a significant interaction between stream and stimulus types ( $F_{3, 73} = 10.84, p = 0.003$ ). Following introduction of alarm cues in Catamaran Brook, salmon reduced their number of feeding attempts, reduced their time spent in movement and increased their time spent on the substrate while such this change in behaviour did not occur in Devil's Brook. I found no repeated measures effect ( $F_{3, 73} = 0.14, p = 0.94$ ) nor any interaction (RM x stimulus,  $F_{3, 73} = 0.14$ ; RM x stream,  $F_{3, 73} = 0.51$ ; RM x stimulus x stream,  $F_{3, 73} = 0.42, p > 0.05$  for all). Overall, these last results suggest that the test subjects were responding in either stream in a similar fashion during both the conditioning and recognition trials.

Taking each stream separately, a repeated measures MANOVA showed that in Catamaran Brook (neutral stream), the type of stimulus used had a significant effect on the alarm response intensity of the test subjects ( $F_{3, 36} = 24.08, p = 0.01$ ) while no repeated measures effect was found ( $F_{3, 36} = 0.11, p = 0.59$ ) nor a repeated measures x stimuli interaction ( $F_{3, 36} = 0.30, p = 0.68$ ). In Devil's Brook (acidic stream) however, a repeated measures MANOVA did not show a significant effect of stimulus type ( $F_{3, 35} =$

0.57,  $p = 0.06$ ) nor a repeated measures effect ( $F_{3, 35} = 0.59$ ,  $p = 0.63$ ) or a repeated measures x stimulus interaction ( $F_{3, 35} = 0.67$ ,  $p = 0.18$ ). These results suggest that although there was a significant difference in the stimulus type used in Catamaran Brook, no such difference occurred in Devil's Brook.

During the conditioning phase, I observed that salmon in Catamaran brook decreased their mean number of feeding attempts and their mean time spent moving while their mean time spent motionless on the substrate increased when exposed to lemon odour paired with the alarm cues compared to lemon odour paired stream water (Figure 4.2). This suggests that the alarm cue exposures elicited an increase in salmon's alarm response. In contrast, in Devil's Brook I did not observed such consistent behavioural changes between stimuli. During the testing phase in Catamaran Brook, I observed similar mean changes in behavioural responses as in the conditioning phase (Figure 4.2), suggesting that salmon had learn to recognize the lemon odour as a potential predation risk when paired with the alarm cue. In Devil's Brook, once again I did not observed any consistent behavioral changes between stimuli suggesting that no learning occurred.

To ensure that these results were not due to differences in baseline activity levels between Catamaran Brook and Devil's Brook as well as between stimulus types, I compared baseline activity rates (before the injection of any stimuli) using repeated measures MANOVAs with streams and stimulus types as independent variables. I found no significant difference in overall baseline activity between the two streams studied ( $F_{3, 98} = 0.82$ ,  $p = 0.49$ ), stimulus types ( $F_{3, 98} = 1.76$ ,  $p = 0.16$ ) nor an interaction of streams x stimulus types ( $F_{3, 98} = 0.31$ ,  $p = 0.82$ ).

As the only environmental difference between the streams was pH (Table 4.1),

differences in behavioral responses could not be attributed to other environmental variables

## **Discussion**

The data demonstrate that under acidic conditions, the ability of wild juvenile salmon to acquire the recognition of a novel odour from chemosensory association with a damage-released chemical alarm cue is impaired (i.e., reduced). In the conditioning phase, the paired alarm cues and lemon odour injections elicited significant changes in alarm response under neutral conditions but not under acidic conditions. In the testing phase, only the individuals initially conditioned with the alarm cues under neutral conditions responded to the novel odour injections. Under acidic conditions, no alarm response occurred. These results concur with the laboratory results obtained by Leduc et al. (2004a, b) and show that if the fish do not detect the alarm cue, they cannot associate a novel chemical cue with it. This demonstrates a potential environmental constraint on this type of learning mechanism.

Because there were no environmental differences across the two streams except in pH, the differences in response may be attributed to pH differences in the environment. Although juvenile fish may be especially vulnerable to ambient acidity, I do not suspect my inability to detect a learned response under acidic conditions to be attributable to a significant physiological stress affecting the test fish. The avoidance threshold for Atlantic salmon is *c.* pH 4.5 (Gunn and Noakes 1986; Peterson et al. 1988; Åltand 1998). Moreover, the baseline scores (before the injection of stimuli) for each behavioural modality measured did not significantly differ between the studied brooks suggesting that

test fish behaved with the same intensity. Alternatively, damage to olfactory receptors may account for the lack of response. Leduc et al. (2004b) have shown that YOY rainbow trout could be conditioned to learn a novel odour when paired with damage-released chemical alarm cues. This learned response however did not occur when the odour was paired with acidified alarm cues (at pH ~ 6.0). In contrast, acidifying the odour had no effect on the retention of this novel learning as long as the paired alarm cue was not acidified. This result suggests that under weakly acidic conditions (pH ~ 6.0), the olfactory function of salmonids is not impaired and cannot explain the lack of alarm response. Lastly, the alarm cues may be degraded (reduced) below a minimum detection threshold due to ambient acidity (Leduc et al. 2003). Several authors have shown that concentrations of alarm cues below some population specific threshold fail to elicit an overt antipredator response (Brown et al. 2001a; Mirza and Chivers 2003; Roh et al. 2004). However, even in the absence of observable alarm responses, chemically mediated acquired recognition of a novel cue may still occur. Brown et al. (2001a) demonstrated that fathead minnows could learn to recognize a predator odour when exposed to concentrations of artificial alarm pheromone below their behavioural response threshold. Such covert responses (not observable during the course of the experiment) could translate into increased survival. Likewise, Mirza and Chivers (2003) conditioned juvenile rainbow trout to alarm cues and the novel odour of an unknown predator (northern pike, *Esox lucius*) with concentrations of alarm cue above and below the trout response threshold. Trout exposed to concentrations below the observable response threshold were able to evade the predator equally as well as trout exposed to alarm cue concentrations above the observable response threshold. As such, these results suggest

that even in the absence of measurable alarm responses, prey may still gain chemosensory information about prevalent predation risks. My current results suggest, however, that the alarm cue is degraded to the point where chemosensory learning no longer occurs. This finding suggests that juvenile salmon could not rely on damage-released chemical alarm cues to gain information about the prevalent predation risks. As such, I argue that acidic conditions impose an environmental constraint on this type of chemically mediated learning. Prey may, however, have access to other sources of chemical information. For example, disturbance-released alarm cues are voluntarily released from prey detecting a predation threat (Wisenden et al. 1995; Bryer et al. 2001). These cues may have a different chemical nature and may not be affected by ambient acidity. More work is required to assess the nature of these disturbance cues and if ambient acidity affects their detection by prey.

The observed loss of alarm function may create a significant loss in risk assessment abilities for juvenile salmon. Under natural conditions, chemically mediated learning is known to result in the rapid acquisition of both visual and chemical predator cues (Brown et al. 1997; Leduc et al. 2007a). Mirza and Chivers (2000) and Darwish et al. (2005) have shown that prey conditioned to recognize novel predator odours exhibit increased survival during staged-encounters with live predators. Under natural conditions, YOY Atlantic salmon are potential prey for many piscivorous fish species, including brook charr, slimy sculpin, and Atlantic salmon parr. These predators may exert significant predation pressures on YOY salmon. In their experiment, Henderson and Letcher (2003) stocked YOY Atlantic salmon into three different brooks. These authors reported that between 4 - 49% of all stocked salmon were successfully preyed

upon and predation mainly occurred within the first two days of stocking. After this initial period, the predation rate on YOY salmon dramatically decreased. These findings support the idea that initially predator-naïve individuals are more easily preyed upon and subsequent acquired recognition of these predators confers increased survival. In the streams studied, I found several fish predator species including brook charr, Atlantic salmon parr and slimy sculpin (A.O.H.C. Leduc, Concordia University, personal observations). I expect that emergent predator-naïve Atlantic salmon fry may learn the identity of their predators from chemically mediated learning and gain survival benefits from this learned response (Mirza and Chivers 2000, 2001a; Darwish et al. 2005). By itself, chemically mediated learned predator recognition should confer significant survival advantages to prey having the ability of making ecologically relevant associations between neutral (novel) and aversive stimuli. Whether the loss of chemosensory learning documented here translates into increased mortality for prey individuals remains unknown.

In many areas affected by acid precipitations, the soil buffering capacity has been heavily depleted by several decades of acid precipitations (Doka et al. 2003). It is estimated that under the current state of sulfuric emissions and the current acid depositions, several decades may be necessary before normal buffering capacity of soil is replenished (Jeffries et al. 2000). Aside from acidity, other sources of anthropogenic pollution may interfere with chemical alarm function. For example, pesticides (Sholtz et al. 2000) and heavy metals (Scott et al. 2003; McPherson et al. 2004) impair the detection of chemical alarm cue. Hence, the range of environmental constraints on chemically mediated learning may be greater than expected.



Given the decline in stocks of salmon (Noakes et al. 2000; Lackey 2003), understanding the effects of acid pollution as an integrated part may help formulate better policies towards salmon sustainability. For example, pre-release antipredator training employed as a management tool to improve the antipredator skills of 'naïve' individuals (Brown and Laland 2001) may benefit from avoiding stocking during periods of increased stream acidification (e.g., after snow and ice meltdown) and as such, may favour increased survival rates of juvenile salmonids.

Table 4.1. Mean value ( $\pm$  standard error) of the physical and chemical variables between Catamaran Brook and Devil's Brook. One-way analysis of variance (ANOVA) was used to assess differences between these brooks for the surveyed variables.

	Catamaran Brook	Devil's Brook	F	df	p
pH	7.15 $\pm$ 0.08	6.08 $\pm$ 0.05	121.37	1, 119	0.001
Dissolved oxygen (%)	96.8 $\pm$ 0.07	95.8 $\pm$ 0.12	1.22	1, 119	0.271
Water temp. ( $^{\circ}$ C)	16.6 $\pm$ 0.11	16.4 $\pm$ 0.09	1.12	1, 119	0.257
Current vel. ( $m \cdot s^{-1}$ )	0.23 $\pm$ 0.17	0.21 $\pm$ 0.26	2.17	1, 119	0.143
Depth (m)	0.20 $\pm$ 0.21	0.23 $\pm$ 0.10	0.002	1, 119	0.969
Width (m)	3.52 $\pm$ 0.72	3.41 $\pm$ 51	0.219	1, 119	0.641
Canopy cover (%)	46 $\pm$ 4	38 $\pm$ 17	3.31	1, 119	0.072
Cloud cover (%)	55 $\pm$ 10	34 $\pm$ 28	11.15	1, 119	0.001
Substrate complexity	88 $\pm$ 0.7	82 $\pm$ 0.13	1.51	1, 119	0.220
Conductivity	91.65 $\pm$ 0.19	25.35 $\pm$ 0.34	2906.02	1,119	0.000

Significance was established when  $p \leq 0.05$ .

Figure 4.1. The location of the study streams in New Brunswick, Canada. The inset map in the top right corner shows the location of the study sites within New Brunswick. The arrows correspond the to study sites (each approximately 100 m in length) located along Catamaran Brook and Devil's Brook.

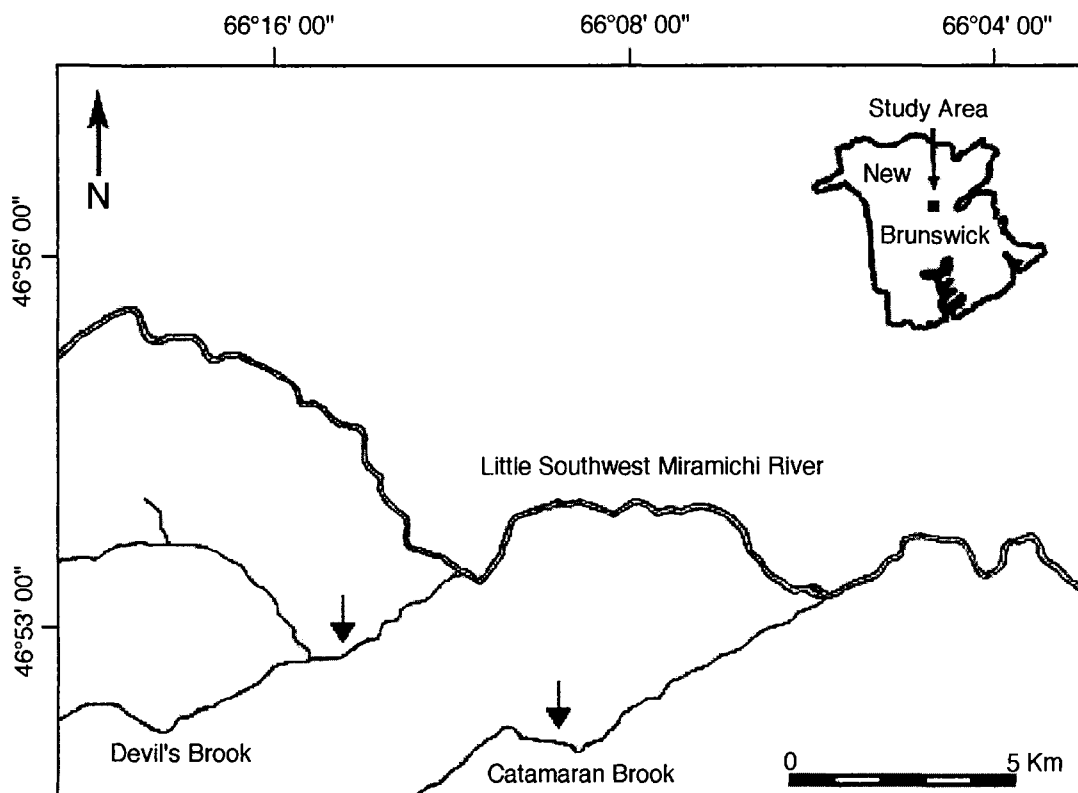


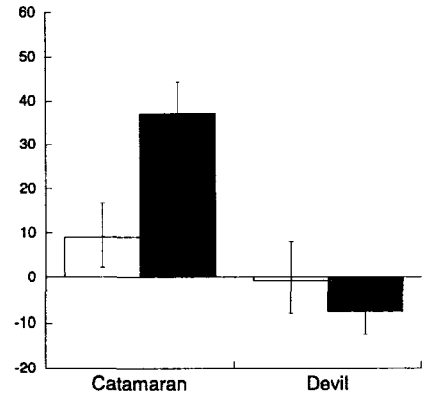
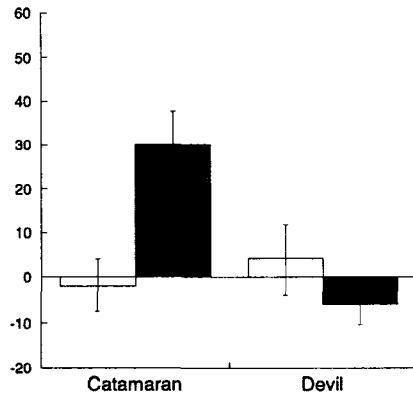
Figure 4.2. Mean ( $\pm$  standard error) differences (post - pre) in: a) time in seconds spent on the substrate, b) number of feeding attempts and c) time in seconds spent in motion for juvenile Atlantic salmon (*Salmo salar*) exposed to either alarm cues (dark bars) or stream water (open bars) under neutral (Catamaran Brook) or weakly acidic (Devil's Brook).

Mean changes in:

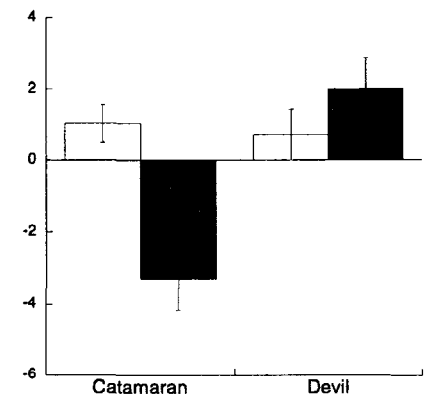
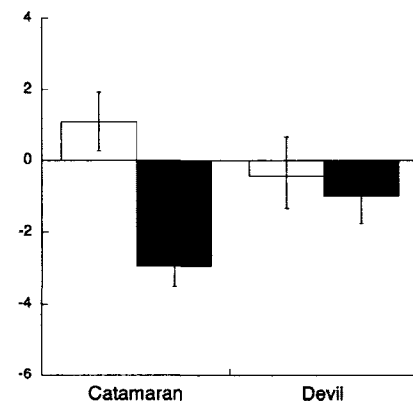
Conditioning phase

Recognition phase

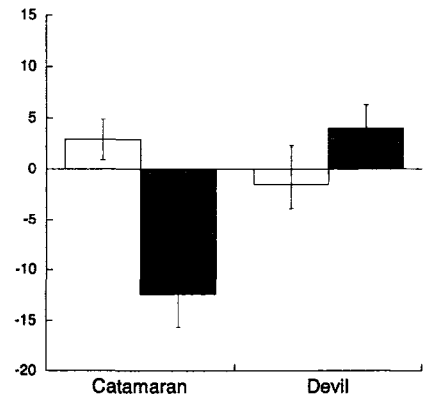
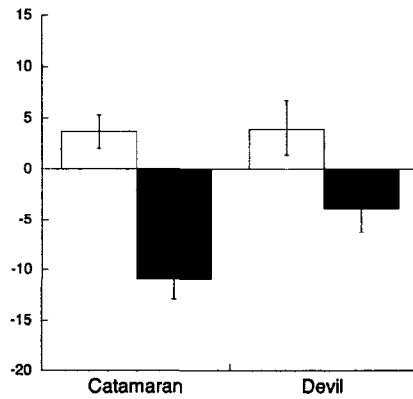
a)



b)



c)



## **Chapter 5. Chemosensory risk assessment loss under weak acidification: evidence of a survival cost from predation.**

### **Introduction**

A wide range of prey fishes including Salmonids, use damage-released chemical alarm cues to assess local predation threats (Brown and Smith 1997; Mirza et al. 2001a; Leduc et al. 2004a). Laboratory and semi-natural enclosure studies have demonstrated the survival benefits of responding to chemical alarm cues during staged-encounters between prey and predator (Mirza and Chivers 2000, 2003). As such, individuals having the ability to detect and respond to these cues should have a selective advantage.

Many anthropogenic chemical compounds released into the environment in sub-lethal concentrations have been shown to disrupt the transfer of chemical information between organisms (reviewed in Lürding and Scheffer 2007). One such chemical disturbance, freshwater acidification, interferes with individuals' ability to use local chemical risk assessment cues. In their experiments, Leduc et al. (2004a, 2006) demonstrated that under weak acidification (pH ~ 6.0), the ability of two species of juvenile salmonid to respond to the chemical alarm cues is significantly reduced compared to neutral conditions (see Chapter 1). Given the demonstrated survival benefits associated with the detection and the behavioural responses chemical alarm cues trigger, low levels of acidification may place prey at increased risk of predation (Mirza and Chivers 2003).

The observed impairment may follow two possible mechanisms. First, acidification may only partially degrade the alarm cues under a minimum response

threshold (Brown et al. 2001a, 2004) in which individuals may gain enough information about local danger to obtain a survival benefit. In staged-encounters between predators and prey, Mirza and Chivers (2003) showed that individuals exposed to concentrations of alarm cues that failed to elicit an alarm response (i.e., sub-threshold concentrations) survived equally as well as prey exposed to concentrations eliciting observable alarm responses. Alternatively, a complete loss of chemical alarm function may occur under acidic conditions (Brown et al. 2002). Under such conditions, prey may be deprived of the information provided by the chemical alarm cues and thus may suffer from an increased survival cost of predation.

Thus, the aim of this study is to determine whether the observed chemosensory loss of the alarm cues under acidified conditions translates into increased mortality for juvenile rainbow trout (*Oncorhynchus mykiss*) in the presence of predatory largemouth bass (*Micropterus salmoides*). This is of particular concern given that each spring, thousands of salmonid fry are stocked into streams that, following snowmelt, become acidified (Dillon et al. 1984; New Hampshire, Fish and Game Department, 2007).

## **Material and methods**

### ***Test subjects***

Young-of-the-year (YOY) rainbow trout were purchased from Piscicultures des Arpents Verts, Ste. Edwidge de Clifton, Québec, Canada. Largemouth bass were captured using beach seine from Lachine Canal Park, Montréal, Québec, Canada. Prior to the experiment, both trout and bass were kept in separate 250-litre recirculating tanks, with a flow rate of 500 mL/minute at a temperature of approximately 16 °C and kept under a



12:12 hours light:dark photoperiod. Trout were fed with commercial trout chow while bass received a combination of frozen brine shrimp and commercial cichlid pellets. Both were fed *ad libitum* daily. Bass and trout were held for a minimum of 30 days before the experiment took place. At the time of the experiment, the mean sizes of the trout and the bass were  $3.72 \text{ cm} \pm 0.18$  and  $8.72 \pm 0.32$  (mean  $\pm$  SD fork length) respectively.

### ***Stimulus preparation***

For this experiment, I used rainbow trout chemical alarm cue produced from skin fillets of 9 YOY rainbow trout donors (mean  $\pm$  SD fork length =  $3.41 \pm 0.18$ ). The chemical alarm cue solution was prepared as in Chapter 1 with  $24.11 \text{ cm}^2$  of skin homogenized in 273 mL of distilled water. The final pH of the alarm cue solution was 6.96. I prepared the alarm cue solution and distilled water (as a control) in 20 mL aliquots and froze them at  $-20 \text{ }^\circ\text{C}$  until needed.

### ***Experimental protocol***

Test trials were conducted in 90 L aquaria (90 x 40 x 33 cm) that were covered with opaque plastic on three sides to avoid visual contact with adjacent tanks and filled with dechlorinated tap water. I equipped each tank with an air stone to which I attached additional airline tubing, allowing for the injection of stimuli without disturbing the test subjects. In each tank, I placed two trout and a single bass that I let acclimate for 24 hours prior to the start of experimental trials. Before starting a trial, both prey and predator were kept in visual isolation by placing the predator in an opaque plastic cylinder of 25 cm in diameter in which dozens of perforations (of approx 4 mm) were

made, allowing fishes to smell their surroundings. Trout were fed approximately 1 hour before trials while bass were starved for at least 24 hours (ranging from 24 -28 hours). The feeding of prey was conducted before trials, as individuals may not respond to chemical alarm when starved (Brown and Cowan 2000).

Nine predators were used in random order for three different stimulus treatments (alarm cues, acidified alarm cues or distilled water). Acidification was achieved by adding approximately 0.4 mL of diluted sulfuric acid ( $H_2SO_4$ ) directly into the 20 mL aliquot of alarm cue stimulus prior to the start of a trial. Adding this volume of acid does not affect the overall tank pH, hence eliminating any physiological stress effects caused by acidification. Before the start of a trial, I withdrew 60 mL of stagnant water from the airline tubing and discarded it. I withdrew and kept an additional 60 mL of water. I injected the stimulus and completely flushed it in with the retained water. I allowed the stimulus to mix in the aquarium for a period of two minutes before starting a trial. At the start of trials, I pulled the cylinder out of the tank and allowed trout and bass to interact until the first trout was captured or 5 minutes had elapsed, whichever occurred first. I quantified the time for first capture (in seconds), the number of bites and the number of chases. Bites were defined as a pecking motion from the predator to the prey while chases were when a predator followed a prey for any distance longer than one body length. These behaviour were used as dependent variables in a repeated measure MANOVA by randomly using each predator once under each stimulus treatment. By doing so, each predator became its own control. I further compared the effect of acidification between treatments using t-tests. All statistical analysis was done using SPSS 11.

## Results

Repeated measures MANOVAs revealed significant effects of stimulus type on the latency to capture ( $F_{2,7} = 10.171$ ,  $p = 0.008$ ; Figure 5.1a). However, no significant difference was observed between treatments for the on number of strikes ( $F_{2,7} = 0.137$ ,  $p = 0.874$ ; Figure 5.1b) and the number of chases ( $F_{2,7} = 0.461$ ,  $p = 0.649$ ; Figure 5.1c) although the observed trend suggests an increased predator success under acidic conditions and distilled water. For the latency to capture, t-tests revealed that no significant difference existed between the acidified and control treatments ( $t_{1,8} = 0.53$ ,  $p = 0.610$ ) whereas the neutral alarm cue produced a higher latency period relative to both acidified alarm cues and distilled water ( $t_{1,8} = 3.593$ ,  $p = 0.007$  and  $t_{1,8} = 3.023$ ,  $p = 0.017$ , respectively).

## Discussion

These results clearly show that acidifying the alarm cues lead to lower survival of juvenile trout. Indeed, the time elapsed before a predator captured a prey following exposure to the acidified alarm cue did not significantly differ with the control treatment while the undisturbed alarm cues treatment lead to longer survival (Figure 5.1a). In 8 of 9 of the alarm cue, the entire trial duration elapsed (5 minutes) during which no trout was captured by a predator while the mean latency to capture in the acidified alarm cue or distilled water treatments were approximately half that of the undisturbed alarm cues treatment. Although no statistical difference across treatments existed for the number of strikes or the number of chases (Figure 5.1b and 5.1c), the observed trends support the idea that under the acidified alarm cues and control treatments, prey are likely to suffer

increased costs linked to predation compared to non-acidified alarm cues. Using a small sample size likely did not allow for sufficient statistical power to make this difference statistically significant as shown in Darwish et al. (2005), while keeping to a minimum the number of individuals to be sacrificed.

The results obtained indirectly suggest that at a pH of 6.0, the damage-released chemical alarm cues are completely degraded and do not provide any information about prevalent risk as suggested by Brown et al. (2002). Such a reduction in pH from circum-neutral to weakly acidic is likely to occur following natural and/or anthropogenic increases in acid inputs (Baker et al. 1996; Komai et al 2002). Therefore, adding to various natural and/or anthropogenic sources of mortality for juvenile salmonids (Ersbak and Haase 1983; Watt 1987), the observed reduced ability to chemically mediate local predation risks may have severe implications under natural conditions. In their experiment, Henderson and Letcher, (2003) showed that after the introduction of predator-naïve hatchery reared juvenile Atlantic salmon in natural streams, predators successfully removed over 60 percent of them within the first week. Relating this finding to management issues, thousand of juvenile salmonids are released each year into North American streams and lakes following snowmelt. As acid in snowmelt can significantly reduce the ambient pH of surface water (Dillon et al. 1984), this may deprive these salmonids of important information on local predation risk.

Indirect changes in predator-prey interactions may create added costs for prey that are chemosensorily impaired. For instance, alarm cues released from injured prey have been shown to attract predators (Mathis et al. 1995). Although it appears to be counterintuitive, such a predator attractant effect has been shown to increase prey

survival, due to multiple predator interference effects (Chivers et al. 1996). While it is not known whether acidified alarm cues can still function as a predator attractant, prey would likely be negatively affected by acidic conditions whether or not the alarm cues serve as a predator attractant. In the affirmative, prey would not be able to rely on the alarm cues under acidic conditions while predators could use them to cue-in onto prey. Alternatively, if the alarm cues no longer attract predators under acidic conditions, captured prey would not be able to use multiple predator interference effects to increase their chance of escaping. The data suggest increased predation risks due to the observed chemosensory impairment that occurs under acidic conditions. Further studies should investigate whether or not such a direct chemosensory impairment loss linked to acidification exists under natural conditions.

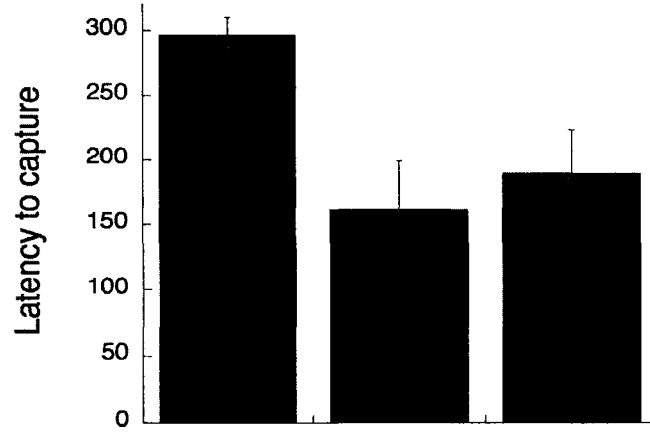
In the same way as for the level of acidity presented in this experiment, other pollutants present in sub-lethal concentrations have been shown to alter prey antipredator behaviour. For example, pesticides created hyperactivity and impaired schooling in Atlantic silversides, (*Menidia menidia*) rendering these prey fish increasingly visible and vulnerable to predation (Little et al. 1990). Likewise, sub-lethal concentrations of heavy metals or pesticides induced sub-optimal schooling behaviour in fathead minnows (*Pimephales promelas*; Atchison et al. 1987) and Atlantic silversides (Weis and Weis 1974). Although these changes do not directly pertain to risk assessment, this together suggests that adaptation potential to the effects of anthropogenic disturbances may be far slower than natural selection could permit. Understanding how anthropogenic changes may affect organisms' chemical information transfer may have far-reaching implications for proper ecosystem functions as well as for adequate management and conservation

policies. This should include the likely ability predator-naïve salmonids would have in surviving predation during the critical post-emergence/stocking period.

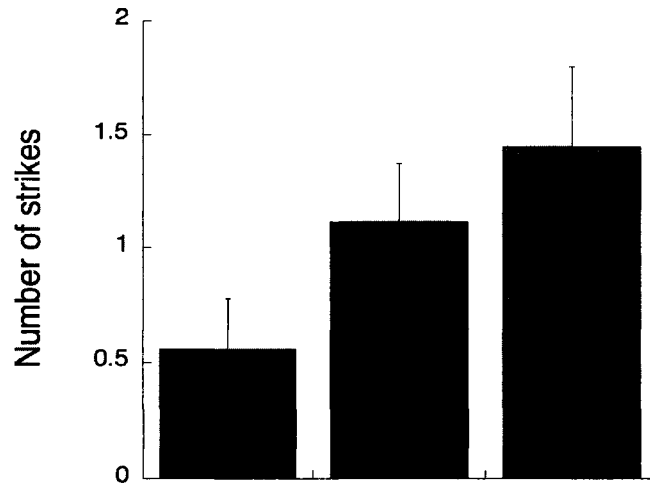
Figure 5.1. Mean ( $\pm$  standard error): a) latency to capture (in seconds), b) number of strikes and c) number of chases by largemouth bass (*Micropterus salmoides*) toward juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to alarm cues (AC), acidified alarm cues (AAC) or distilled water (DW).

Mean results

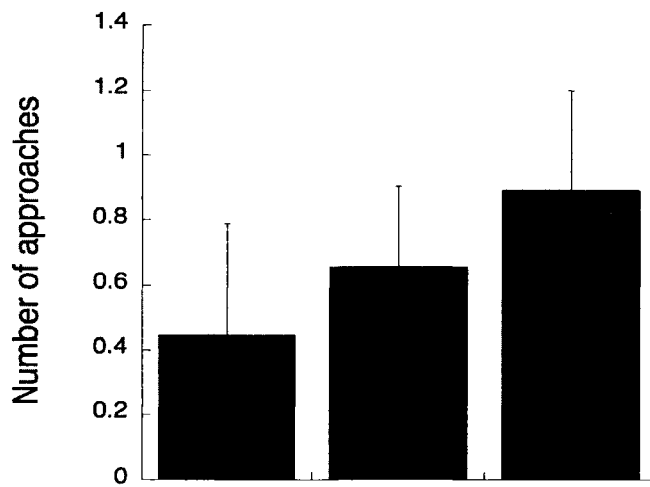
(a)



(b)



(c)



AC AAC DW

Stimulus treatments



## **General Conclusions**

The results presented in this thesis suggest that even subtle increases in ambient acidity may interfere with the use of chemical alarm cues in otherwise pristine conditions.

Chapter 1 shows no significant variation in chemosensory impairment in juvenile Atlantic salmon in their natural habitat across two groups of streams different in their acidity levels. For instance, under acidic conditions, the intensity of the alarm response is similar for the two age classes and all populations while fluctuations in alarm responses were shown to occur under neutral conditions (neutral streams). Also, the intensity of the alarm response is consistently reduced under acidic conditions compared to neutral conditions. These findings support the idea that differences in alarm response intensity did not vary because of population differences in acid tolerance but rather because of a chemical effect occurring to the chemical alarm cues under acidic conditions.

Chapter 2 further suggests that chemosensory impairment to alarm cues in natural conditions does not likely result from population differences in the production or the detection of the chemical alarm cues, but is rather created by ambient acidity. This statement is at least valid when considering populations of freshwater fish found in the same drainage basin or when environmental conditions, food abundance and predator pressures are similar. In drastically dissimilar conditions, sufficient population differences may start to occur leading to differences in chemosensory assessment. In this study, I did not find any difference in the ability to respond to the alarm cues between salmon populations initially found under neutral or acidic conditions. The main correlate of chemosensory detection appears to be the level of ambient acidity in which the test subjects were tested. Likewise, I did not find any significant difference in the 'potential'

to trigger alarm behaviour between chemical alarm cues from different salmon populations. When tested under neutral conditions, all the different alarm cues elicited similar alarm response intensity. Again, these results suggest that the alarm response to chemical alarm cues under natural conditions is dependent on the ambient pH.

Chapter 3 shows the acidity threshold for the loss of detection of the chemical alarm cues to occur between a pH 6.4 and 6.2. At pH 6.2, no observable alarm response occurs while a reduced response was still observable at pH of 6.4. This study also shows that the loss of response is graded, in which the intensity of the alarm behaviour diminishes with decreasing acidity suggesting that prey individuals may gain access to partial information provided by the alarm cues. Although these results were found under laboratory conditions, the source of acidity used (sulfuric acid) could be similar as to the source of acidification from anthropogenic sulfuric emissions. If extrapolated to natural conditions, these results suggest that even minor reductions in ambient acidity (from pH 7.0 to pH ~ 6.4) may occur in a cyclic/seasonal manner resulting in a significant reduction of alarm behaviour from a chemosensory function loss.

Chapter 4 shows that acquired association of chemical alarm cues with a novel odour does not take place under acidic conditions. Indirectly, this study suggests that alarm cues are degraded under a minimum behavioural threshold as no recognition occurred when 'pseudo conditioned' individuals (individuals conditioned under acidic conditions) were tested for the recognition of the novel odour. This result suggests that under episodic acidification, the learning of novel predator may not be facilitated from chemosensory function. However, other types of learning may still occur even when the chemical alarm cues are no longer functional. For instance, visual learning can be

reinforced between a novel stimulus (novel odour) and a visual stimulus (such as alarm behaviour). Such 'cultural' conditioning has been shown to lead to the acquisition of long-term retention of a novel cue as a potential hazard. It is not known however, to what extent prey individuals may be relying on cultural transmission or other visual cues when deprived from chemosensory learning.

Finally, chapter 5 underscores the ecological relevance of the loss of chemical alarm cues detection occurring under acidic conditions. The most relevant measure of survival (time until capture) was significantly shortened when chemical alarm cues were acidified. Other measures however failed to show significant effects of acidification on the survival parameters studied. Although these non-significant results may be due to the small sample size used, they follow the predicted trend in which the cost to individual prey was higher when no chemosensory risk assessment was available.

Taken together, these results highlight the importance of assessing individuals' behavioural changes under various sets of conditions. Indeed, this may reveal that under certain conditions, behavioural changes may be sub-optimal and potentially lead to increased fitness costs for individuals. For instance, the level of acidity in which the experiments were conducted was not believed to create significant physiological stresses or behavioural changes in many salmonid species. The observed chemosensory risk assessment impairment shown in this study may have consequences for the survival of juvenile salmonids, especially shortly after their emergence, as this could lead to lower over-summer survival and potentially, to population decline. However, as different sensory modalities may be used to assess local predation risk, the observed chemosensory impairment may be compensated for by increased visual assessment or reliance to other

type of chemical cues. These possibilities deserve attention and may lead to interesting findings.

The knowledge that many wild salmonid species have been drastically reduced in population number should further the interest in providing ecologically adequate environmental conditions. Having a thorough understanding on the global effects of anthropogenic impacts is a necessary step to succeed at managing and protecting sensitive aquatic biota.

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