Direct and indirect effects of predation on juvenile Chinook (*Oncorhynchus tshawytscha)* salmon

Lauren Kuehne

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Julian Olden, Chair

David Beauchamp

Thomas Quinn

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Table of Contents

Chapter I: Prey naivety in the behavioural responses of juvenile Chinook (*Oncorhynchus tshawytscha*) salmon to an invasive predator

Chapter II: Costs of living for juvenile Chinook salmon in an increasingly warming and invaded world

Chapter I: Prey naivety in the behavioural responses of juvenile Chinook (*Oncorhynchus tshawytscha*) salmon to an invasive predator

Abstract

Non-native predators might inflict proportionally higher mortality on prey that have no previous experience of them compared to species that have coexisted with the predator for some time. We tested whether juvenile Chinook salmon (*Oncorhynchus tshawytscha*) were less able to recognise a non-native than a native predator, by investigating behavioural responses to the chemical cues of the invasive smallmouth bass (*Micropterus dolomieu*) and the native northern pikeminnow (*Ptychocheilus oregonensis*) in both laboratory and field experiments.

Laboratory results demonstrated strong innate antipredator responses of individual juvenile Chinook salmon to northern pikeminnow; fish spent 70% of time motionless and exhibited 100% greater panic response than in controls. By contrast, antipredator responses to the chemical cues of smallmouth bass did not differ from controls. These results were supported by similar differences in recognition of these predator odours by groups of juvenile Chinook salmon in fully natural conditions, though responses reflected a greater range of antipredator behaviours by individuals. In field trials, responses to northern pikeminnow odour resulted in increased flight or absence, reductions in swimming and foraging, and increased time spent near the substratum, compared to smallmouth bass odour.

Given that survival of juvenile fish is facilitated by predator recognition, our results support the hypothesis that naivety may be an important factor determining the effect of non-native predators on prey populations. Efforts to manage the effect of native and non-native predators may benefit by considering complex behavioural interactions, such as these at the individual and group levels.

Introduction

Human-assisted dispersal of non-native species has caused widespread changes in the global distribution of organisms (Olden *et al.*, 2004; Hobbs *et al.*, 2006). The ecological effects of non-native species on recipient ecosystems have ranged from positive to severely negative (Parker *et al.*, 1999; Mack *et al.*, 2000; Schlaepfer, Sax & Olden, 2011), spawning a suite of hypotheses to explain this variability. From the standpoint of the invader, important factors determining impact include characteristics of dispersal (Wilson *et al.*, 2009), release from enemies or pathogens, and adaptation to or suitability in human environments (Sax & Brown, 2000). In the invaded range, factors associated with impact include biotic resistance (Fridley *et al.*, 2007), alteration of habitat (Sax & Brown, 2000) and inability of native species to cope with characteristics of novel invaders (Sih *et al.*, 2010). Of these, reduced or ineffective responses by native species to novel predators due to a lack of prior experience (also known as 'prey naivety') has been identified as a possible contributor to the decline of the former (Schlaepfer *et al.*, 2005). Research on a range of taxa including mammals (Banks, 1998; Fey *et al.*, 2010; Barrio *et al.*, 2010), invertebrates (Freeman, 2006; Edgell & Neufeld, 2008), amphibians (Pearl *et al.*, 2003; Gomez-Mestre & Diaz-Paniagua, 2011) and fish (McLean, Barbee & Swearer, 2007) has demonstrated reduced recognition, less effective defences or increased mortality in response to non-native than to native predators in laboratory experiments. Prey naivety has also been hypothesized to play a particularly important role in freshwater environments due to spatial habitat heterogeneity and barriers to dispersal, which promote adaptation to localized predation regimes and the potential for greater vulnerability to introduced predators (Cox & Lima, 2006).

Recent research demonstrates that naive prey do not respond consistently differentially to, or suffer higher mortality from, non-native predators (Rehage, Dunlop & Loftus, 2009;

Kovalenko *et al.*, 2010). Some studies have suggested that the ability to respond to novel predators is predominantly driven by factors such as level of predation risk in a system (Murray, Roth & Wirsing, 2004), similarity of non-native predator behaviour or morphology to that of native predators (Rehage *et al.*, 2009), or reliance of prey species on learned versus innate systems (Epp & Gabor, 2008). Ambiguity in past research findings may be partly due to experimental limitations on prey naivety, which ultimately demands demonstrating increased vulnerability to predation in ecologically relevant settings. Laboratory settings typically suffer from a 'fence effect', where prey are behaviourally constrained, while experiments which use wild organisms are unable to control for differences in experience and learning. Native and non-native predators used in comparative studies may also have subtly different hunting modes, making comparisons of prey behavioural response and subsequent mortality challenging (Rehage *et al.*, 2009). Prey populations in the wild inevitably have access to additional chemical, visual or social cues that may mediate effects of naivety, but can be difficult to fully replicate in laboratory settings. For these reasons, there are recognised gaps in research between laboratory and natural environments, and difficulties in assessing the relevance of prey naivety at a population level (Sih *et al.*, 2010). We investigated the innate behavioural responses of juvenile Chinook salmon (*Oncorhynchus tshawytscha* Walbaum) to the chemical cues of native northern pikeminnow (*Ptychocheilus oregonensis* Richardson) and non-native smallmouth bass (*Micropterus dolomieu* Lacepède) predators in laboratory and field environments to compare responses in controlled and fully natural settings.

Our choice of prey and predator species reflects not only the significant management interest in the impacts of predation on endangered juvenile Pacific salmon, but the relatively comprehensive knowledge of the biology, evolutionary history and distributions of these species in the Pacific Northwest region of North America. The northern pikeminnow is a native cyprinid found in lentic and lotic systems from northern Oregon to British Columbia (Wydoski & Whitney, 1979; Li *et al.*, 1987); one of the largest members of the minnow family native to North America, it has been identified as a significant predator of emigrating salmon smolts in the Columbia River hydropower systems and has been actively controlled for two decades (Tabor, Shively & Poe, 1993; Zimmerman, 1999). The smallmouth bass was chosen as a highly invasive predator that is of concern, due to its widespread distribution (Vander Zanden *et al.*, 2004; Sharma *et al.*, 2007) and well known negative effects on the diversity and abundance of native fish (MacRae & Jackson, 2001; Jackson, 2002). The first known introductions of smallmouth bass from eastern North America to the Pacific Northwest were in the 1920's; subsequent decades saw continued stocking by state agencies and private citizens (Carey *et al.*, 2011). Smallmouth bass have become an increasingly important predator of juvenile salmon over the last two decades (Sanderson, Barnas & Rub, 2009). Although estimated predation rates vary across the region, analysis over several emigration periods (March – June) suggests that smallmouth bass predation could account for between 4-35% of wild fall-run Chinook smolts in the lower Yakima River, depending on environmental conditions (Fritts & Pearsons, 2004). However, juvenile Chinook salmon are unlikely to have evolved chemosensory recognition of smallmouth bass. One study (involving frogs) has showed development of innate predator recognition after 20 generations under heavy selection pressure (Kiesecker & Blaustein, 1997) whereas others have not found this in a similar number of or more generations (Paoletti, Olson & Blaustein, 2011; Gomez-Mestre & Diaz-Paniagua, 2011).

We hypothesized that juvenile Chinook salmon would show an innate recognition and response to the chemical cues of northern pikeminnow but not to smallmouth bass. We first tested responses of individual juvenile salmon in a laboratory, using two channel choice flumes. A similar chemical cue experiment was later conducted with groups of wild Chinook salmon under fully natural field conditions, to test the validity of the laboratory results and examine the ecological implications of any observed differences in behaviour.

Methods

Experiment 1: Chemosensory recognition of predators under laboratory conditions

Study organisms

Spring-run (Yakima River population) Chinook salmon eggs were acquired by the Cle Elum Supplementation Research Facility from 15 families of natural origin spawned during September 2009. The eggs were transferred to the Northwest Fisheries Science Centre (National Oceanic and Atmospheric Administration) two months later, and incubated at 5°C until they began feeding in mid-February 2010. In early April, the fish (approximate fork length 40 mm) were transported to the Western Fisheries Research Centre (WFRC; United States Geological Survey), and reared in sand-filtered and UV-treated fresh water from Lake Washington at ambient intake temperatures (mean ± SD = 10.1 ± 1.0 °C) in a 700-L circular tank. Juvenile Chinook salmon were held on a natural photoperiod regime and fed twice daily *ad libitum* (Bio Oregon bioVita) until the trials began in early June.

Smallmouth bass (n = 3; fork length: mean ± SD = 390 ± 10 mm; mass: mean ± SD = 1018 ± 85 g) and northern pikeminnow (n = 5; fork length: mean ± SD = 398 ± 27 mm; mass: mean ± SD = 812 ± 192 g) were captured from Lake Washington, and housed in 700-L circular tanks at WFRC. Predators were closely matched in biomass, as juvenile salmon have a sophisticated ability to distinguish between predator concentration as well as low and high risk predators based on chemical cues (Vilhunen & Hirvonen, 2003; Hawkins, Magurran & Armstrong, 2007). Northern pikeminnow and smallmouth bass in our targeted size class (350-400 mm) are effective and efficient predators of juvenile Chinook salmon in the size range tested (Tabor *et al.*, 1993; Zimmerman, 1999); this was corroborated by our own separate predation trials (L. Kuehne, *unpublished data*). Predator diets were restricted to earthworms for 7 days prior to experiments to control for juvenile salmon responses to diet cues in predator faeces.

Experimental protocol

Two channel choice flumes were used to test avoidance and preference of fish to chemical cues of different predator species. The flumes were constructed from opaque acrylic (20 cm *h* x 30 cm *w* x 90 cm *l*) with a clear acrylic wall (20 cm *h* x 60 cm *l*) dividing the channels. The inflow areas were separated by a white plastic screen, creating a 5 x 15 cm mixing zone for incoming water, and a white mesh plastic sliding gate created a 17 x 25 cm acclimation (pre-choice) area at the rear of the flume (Fig. 1). The outflow pipe in the rear was screened off, and water levels were set to a depth of 10 cm. Four flumes were arranged side-by-side, with two video cameras (Q-See Model QR208-811, 3.6 mm lens) positioned overhead to record trials, allowing the operator to remain out of sight. The tanks were set on rubber pads to reduce influence of vibration and surrounded by black plastic sheeting; incident light was kept low (mean ± SD = 42 ± 14 lux) to reduce shadowing.

Water was gravity-fed to individual channels from 75-L ‘stimulus’ tanks containing either two pikeminnow, or two smallmouth bass, or one pikeminnow and one smallmouth bass, or nothing (control). Each day, these predator pairs were moved to stimulus tanks one hour prior to the start of the first trial, and were removed at the end of the day. Water was delivered to the channels at 1 L min^{-1}, resulting in residence time of ~40 min in stimulus tanks (which were aerated to compensate for changes in oxygen from predator respiration). Stimulus tanks always contained the same predator species or combination; tanks were drained at the end of each day, cleaned with Argentyne-solution and refilled overnight.

To reduce variations in behaviour due to hunger, Chinook salmon were fed *ad libitum* by hand every 3 h on trial days. For each trial, we placed a single salmon in the pre-choice area of each flume and, after allowing 20 min acclimation time, water lines with treatment or control water were connected to each channel. Pre-trial dye tests revealed that incoming water reached the pre-choice area after approximately 2 min (mean ± SD = 2.2 ± 0.6). Accordingly, the gates were raised remotely, using a pulley system of fluorocarbon fishing line, two minutes after the water lines were connected, allowing the salmon to enter the channels. All trials were recorded by video from the time of placement into the pre-choice area to the end of the trial (10 or 15 min, see below). Juvenile salmon averaged 65 ± 5 mm (fork length: mean ± SD), and individual fish were used only once.

We analysed the video recordings to quantify three behavioural metrics over discrete time intervals after the gate was raised: number of transits, activity and channel occupancy. 'Transits' were counted as movement across sections within the tank, as determined by a grid overlay (Fig.1), summed every 20 seconds for comparison over time (i.e. 30 time points for 10 min trials). 'Activity' was characterized every 20 seconds using a modified movement index (Johnsson, Höjesjö & Fleming, 2001) which described: a) movement of fins or body without displacement, b) swimming, c) no motion (hereafter 'freezing') and d) swimming burst or darting response (hereafter ‘panic’). Of these behaviours, freezing and panic are routinely used as indicators of antipredator response in juvenile salmonids (Healey & Reinhardt, 1995; Vilhunen & Hirvonen, 2003; Scheurer *et al.*, 2007). ‘Channel occupancy’ was assessed as the channel where the fish was located at the end of each 20 sec interval. Because responses of individual fish were very similar over short time periods (e.g. a fish that was frozen remained so for many minutes or a fish that was swimming comfortably continued that response over time), our use of discrete time intervals gave a good representation of the amount of time spent in each behaviour; therefore, for ease of reference, we hereafter report the percent of intervals as ‘percent time’. The observer quantifying the behavioural metrics was blind to the treatment.

We conducted 24 replicates over 10 min trials of four odour combinations (treatments): (1) bass:control, (2) pikeminnow:control, (3) bass:pikeminnow, (4) bass+pikeminnow:control. In addition, we performed 36 control trials (control:control) and an additional 18 replicates of treatments (1) and (2) over 15 min. This allowed control trials to be later truncated for comparison with 10 min trials, or used in full for comparison against the additional replicates of treatments (1) and (2) over the longer 15 min time period. Flumes were drained and cleaned between trials with Argentyne-treated brushes and control water. In addition, treatments were assigned to a different flume each day and the treatment channel switched at midday to allow testing for the effect of flume position and treatment channel.

Statistical analysis

The behaviour of each juvenile Chinook salmon was summarized for each 10 min trial using three metrics: percent of time frozen, percent of time exhibiting panic behaviour and percent of time in the stimulus channel (note: the stimulus channel was randomly assigned for the control:control treatments. As the bass:pikeminnow treatment contained a potential stimulus in both channels, we defined pikeminnow as the stimulus in that treatment). To meet assumptions of normality and heteroscedasticity, all variables were arcsine transformed prior to conducting separate ANOVAs. Although some slight departures from normality existed within some treatment groups following data transformation, ANOVA is considered robust to departures given sufficient sample sizes and equality of variance (Zar, 1999; p.185). *Post-hoc* comparisons were made using one-tailed Dunnett's test against the control treatment and Tukey's HSD between other treatments. To examine responses over time, time series of mean and variance in number of transits were generated by treatment (15 min trials only). Smallmouth bass predators remained healthy throughout trials, but two northern pikeminnow had to be replaced; potential consequences of using different predators were therefore tested with no statistical difference in the effect on any behaviours (ANOVA, $P > 0.3$ for all variables). There being also no effect of flume position or treatment channel (ANOVA, $P > 0.4$), data from all of these groups were pooled in subsequent analysis.

Experiment 2: Chemosensory recognition of predators under natural conditions

Study organisms and sites

We tested for chemosensory recognition of predators by juvenile Chinook salmon in Lower Swauk Creek (47.32 N, 120.68 W); a third-order tributary to the upper Yakima River, Kittitas County, Washington State (Fig. 2). Lower Swauk Creek is privately owned and minimally disturbed, and contains a wild population of Chinook salmon. An initial survey prior to the experiment identified sites with at least 6 (mean ± SD = 10 ± 4) juvenile Chinook salmon visible to a snorkeler holding position in the stream, ensuring that video footage taken for subsequent analysis would allow two focal follows per site. Adult northern pikeminnow and smallmouth bass are present in the mainstem Yakima River, but have not been seen in Swauk Creek (Pearsons *et al.*, 1994, G. Temple, Washington Dept. of Fish and Wildlife (WDFW) Ecological Interactions Team, personal communication).

Predator stimuli

Four days before the experiment, two northern pikeminnow (fork length: 330 and 380 mm) and two smallmouth bass (fork length: 300 and 310 mm) were collected using electrofishing and angling from the mainstem Yakima River. Following capture, predator fish were held at the Yakama Nation Hatchery in Prosser, WA and deprived of food for 72 h to decrease the possibility of a reaction by Chinook salmon to diet-related cues. This is a shorter holding period than predators in the laboratory, but should still ensure gut evacuation (Rogers & Burley, 1991). To replicate laboratory conditions as closely as possible, predator odour was prepared by placing the two individuals of each species in 75-L plastic tubs of well water for one hour. Predator water was collected in 4-L containers for transport on ice in insulated coolers, and well water with no predator odour was collected for control treatments. There is little information on the length of time predator odour remains viable in storage, therefore the experiment was conducted as quickly as possible and the ice refreshed daily to maintain constant temperature. The last trial with predator-scented water was conducted within 48 hours of water collection.

Experimental protocol

The experiment was carried out between 21 and 23 September 2010 by a two-person team consisting of a snorkeler and an observer. Predator odour treatments (northern pikeminnow, n = 6; smallmouth bass, n = 6; and control, n = 3) were determined by the observer so that treatments were alternated over time and space without bias by the snorkeler. Approaching from downstream, the snorkeler installed a clear 13-mm nylon hose approximately 1 m upstream of the observed fish. Installation of the hose did not have a noticeable effect on the abundance of juvenile Chinook salmon in a pool or the area in which they were distributed; only one site was abandoned because fish were sufficiently disturbed that they vacated the pool.

After allowing 10 min for acclimation, the snorkeler re-entered the pool with a small hand-held camera (Go-PRO HD Surf HERO) and slowly moved into a position 45° downstream and to the side of the main school of fish. Once the snorkeler was in position, the observer started the experiment. Every minute the snorkeler was cued to count fish in the pool; after 10 min the observer dispensed treatment water through the hose over a 20 - 40 sec period. Counts were recorded for the 10 min following (min 11-20), at which point the experiment was terminated. Pool depth, length and velocity were measured, and the dominant substratum and other species abundances recorded; dye tests conducted at three sites determined the approximate residence time of the 4-L volume of treatment water added to pools (mean ± SD = 69 ± 4 sec). Analysis of environmental variables indicated no significant differences in flow, temperature or pool size between treatment groups (ANOVA, $P<0.18$ for all variables).

We later used the video recordings to conduct focal follows on two randomly selected fish per site using standard media player software (Quicktime Pro). Juvenile salmon in Swauk Creek exhibited the territorial feeding behaviour common in stream-dwelling salmonids (Quinn, 2005), allowing relative ease in focal follows of individuals before and after the stimulus. Without knowledge of the treatment, an observer measured the following behavioural variables for 10 minutes before and after introduction of predator cue: (1) time absent from a feeding territory, (2) time on the substratum, (3) number of feed strikes and (4) time spent swimming.

Focal follows could not be conducted for one pikeminnow site due to poor video quality. During video analysis it also became clear that at one smallmouth bass site the observer was visible to fish, so that site was removed from all analysis; this resulted in a final *n* of six, five and three snorkel observations of pikeminnow, smallmouth bass and control, respectively, and five, five and three for focal follow data.

Statistical analysis

We tested antipredator responses by subtracting mean pre-stimulus from post-stimulus scores for all variables, resulting in difference scores between the two periods; statistical tests were then conducted on difference scores with predator stimulus as a factor. The change in fish observed (based on snorkeler observation) was converted to a percent increase or decrease to standardize for differences in abundance between sites; treatment effect was tested on this variable using one-way ANOVA.

As fish received the same stimulus at each site, scores for the two focal follow fish were averaged for a site score per variable. If a fish perceived a predator threat, we expected an increase in time absent, a shift in water column position toward the substratum, reduced feeding and reduced swimming activity (Berejikian, Tezak & LaRae, 2003; Leduc *et al.*, 2007). Given expected correlations among these responses, multivariate analysis of variance was used to test an overall effect of stimulus type, with subsequent analysis of variance tests on individual variables. The assumption of normality was not consistently met by all variables, therefore, we used permutational analysis of variance (PERMANOVA; Anderson, 2001) with 9,999 permutations for all multivariate and univariate tests of global significance; PERMANOVA as implemented by the Vegan package also has the advantage of allowing testing of unbalanced designs (Oksanen *et al.*, 2009). Significant pairwise differences were determined with Tukey-Kramer *post-hoc* tests.

Results

Innate recognition and response in the laboratory

Juvenile salmon spent significantly more time frozen and exhibiting panic responses in the presence of native northern pikeminnow odour compared to the control (Fig. 3a-b; $P < 0.03$ for both variables, ANOVA with Dunnett's *post-hoc* test) whereas no differences were observed between non-native smallmouth bass and control treatments ($P > 0.38$ for both variables, ANOVA, Dunnett's). All treatments containing pikeminnow, whether alone (Dunnett's, freeze: $P < 0.001$, panic: $P = 0.01$), combined with bass (Dunnett's, freeze: $P < 0.001$, panic: $P = 0.03$), or as alternative to bass odour (Dunnett's, freeze: $P < 0.001$, panic: $P = 0.01$), differed significantly from controls, but not from each other (ANOVA, Tukey's, $P > 0.98$ for both variables). There was no statistical effect of treatment on the percent of time spent in the stimulus channel (Fig. 3c; ANOVA $F_{4,127} = 1.68$, $P = 0.16$).

Behavioural responses of juvenile salmon varied over time across treatments, as shown by the time series of transits during the 15 min trials (Fig. 4). There was minor variation in response to the gate opening in control and smallmouth bass treatments, but response to pikeminnow odour was marked by an initial period of very high transit rates followed by long periods of immobility; short bursts of panic and high transits were often repeated later in the trial (Fig. 4a). This difference in patterns of panic and immobility was further reflected in the time series of standard deviation in transits (Fig. 4b); smallmouth bass and control treatments exhibited similarly moderate variation in the first three minutes, which stabilized over time, whereas responses to northern pikeminnow showed much greater overall variation.

Recognition and response in a natural stream

In the Lower Swauk Creek experiment, the percentage of fish visible to the snorkeler decreased in northern pikeminnow treatments and increased in smallmouth bass and control treatments following introduction of predator or control odour (Fig. 5; PERMANOVA, $F_{2,11} = 3.63$, $P = 0.06$). There was a significant treatment effect (PERMANOVA, $F_{2,10} = 3.21$, $P = 0.03$) on the overall behaviour of focal fish (combination of time absent, time on the substratum, feed strikes and time swimming). Subsequent PERMANOVA analysis on the individual variables showed a significant increase in time spent on the substratum (Fig. 6b; $F_{2,10} = 4.81$, $P = 0.03$) and reduction in time swimming (Fig. 6d; $P = 0.045$) in northern pikeminnow treatments. Though not statistically significant, visual inspection of time absent and feeding strikes showed increased flight or hiding and reduced foraging in response to northern pikeminnow odour (Fig. 6a, c; $P > 0.07$). Response to inputs of control and bass odour resulted in consistently opposite or neutral trends from northern pikeminnow odours, including a tendency toward increased visibility, swimming and foraging (Fig. 6a-d).

Discussion

Our study supported the prey naivety hypothesis by demonstrating a strong difference in innate responses of juvenile salmonids to a native versus non-native predator. Although similar results have been witnessed in other taxonomic groups (mammals: Banks, 1998; Fey *et al.*, 2010; Barrio *et al.*, 2010, invertebrates: Freeman, 2006; Edgell & Neufeld, 2008, amphibians: Pearl *et al.*, 2003; Gomez-Mestre & Diaz-Paniagua, 2011) and, to a lesser extent, other fish species (McLean *et al.*, 2007), our study is unusual in testing these responses in both laboratory and field settings to reflect more accurately the potential relevance of naivety under natural conditions. In aquatic systems, despite the existence of innate chemical recognition of native predators being reported for some species (reviewed in Ferrari, Wisenden & Chivers, 2010), this is not considered a universal phenomenon. On the contrary, aquatic species are understood usually to require learning of predator odours (Ferrari *et al.*, 2010). Recent studies have shown that aquatic prey can acquire predator recognition in the egg stage (Ferrari & Chivers, 2009), raising questions as to whether prey from wild eggs are actually naive. In our study, wild parents spawned in a hatchery upstream of populations of northern pikeminnow or smallmouth bass. While tests of innate recognition must carefully consider prey origins, we believe our study considerably strengthens the case for the existence of innate responses in some species.

Predator-naive juvenile Chinook salmon exhibited a strong antipredator response to the chemical cues of northern pikeminnow but failed to respond to the odour of an introduced centrarchid predator (smallmouth bass). Previous research testing response of aquatic species to chemical cues of native and non-native predators have found differences in species of frogs (Pearl *et al.*, 2003; Bosch *et al.*, 2006; Gomez-Mestre & Diaz-Paniagua, 2011), salamanders (Murray *et al.*, 2004; Gall & Mathis, 2010), fish (McLean *et al.*, 2007) and crayfish (Shave, Townsend & Crowl, 1994). However, generalizing these findings is difficult because yet other studies have failed to find definitive innate differences, including for frogs (Murray *et al.*, 2004; Smith *et al.*, 2008), fish (Kovalenko *et al.*, 2010) and crayfish (Gherardi *et al.*, 2011). It is most informative in this context to compare our results with studies which also used fish prey, as processing and use of chemical cues are likely to be more similar among vertebrates that inhabit similar environments (Derby & Sorensen, 2008). Kovalenko *et al.* (2010) found no difference in the response of tetras (*Hemigrammus marginatus* Ellis) to recently introduced peacock bass (*Cichla kelberi* Kullander and Ferreira) and native wolfish (*Hoplias malabaricus* Bloch). However, prey were captured in the wild, and prior experience or learning could not be controlled. In addition, it is unclear how predator diets were controlled prior to experimental trials, which may have resulted in similar recognition. Our results are more consistent with those of McLean *et al.* (2007), who found a strong antipredator response of both newly recruited and juvenile galaxiids (*Galaxias maculatus* Jenyns) to odours of native eel predators (*Anguilla australis* Richardson*)*, but only mild avoidance to the odour of introduced rainbow trout (*Oncorhynchus mykiss* Walbaum).

Our study further revealed that similar treatment effects were found even in the dynamic environment of a rearing stream. Both group and individual fish behaviours showed a coordinated trend in northern pikeminnow treatments toward absence from feeding territories, reduced swimming and feed strikes, and shifting to a benthic position. These results are consistent with those of Berejikian *et al.* (2003), who tested the response of juvenile Chinook salmon to northern pikeminnow in flow-through aquaria with a gravel substratum. Although assays were done with individual fish, they reported similar changes in reduced activity, feeding and slightly increased time near the substratum. By contrast, fish in our control and smallmouth bass trials generally increased activity and visibility; this is a reasonable result given that, unless a predator threat was perceived, one might expect increasing acclimation to the snorkeler over time. Leduc *et al.* (2007) witnessed a similar slight increase in activity and visibility by juvenile Atlantic salmon (*Salmo salar* Linnaeus) in control treatments of a natural stream experiment. It is also possible that a pulse of new water attracted fish as a potential source of food or by stirring up detritus.

Although our study demonstrated a strong difference in innate recognition and suggested its adaptive value (flight and increased caution), we were not able to test the survival implications explicitly. To our knowledge, only three studies so far have tested explicitly the relationship between innate recognition of chemical cues and increased survival in direct predator trials. Kovalenko *et al.* (2010) found no difference in recognition or behaviour by tetras in response to chemical cues of a native and non-native predator, but were unsuccessful in testing differential mortality, as predators did not actively pursue prey. By contrast, Kiesecker & Blaustein (1997) found reduced response in allotopic versus syntopic populations of red-legged frog (*Rana aurora* Baird and Girard) to chemical cues of introduced bullfrogs (*Rana catesbeiana* Shaw) resulting in increased rates of predation. Similarly, Gomez-Mestre & Diaz-Paniagua (2011) reported that Iberian green frogs (*Pelophylax perezi* Seoane) failed to exhibit induced antipredator defences in response to invasive crayfish (*Procambarus clarkii* Girard), and also suffered significantly higher mortality compared with a native dragonfly (*Anax imperator* Leach) predator. While not precisely parallel, chemical ecology and aquaculture research contain examples of survival studies of naive fish trained to recognise novel predator odours (Mirza & Chivers, 2000; Gazdewich & Chivers, 2002; Vilhunen, 2006). These and other studies, reviewed in Ferrari *et al.* (2010), consistently associate higher survival with advance warning and greater predator recognition. Although our study does not address differences in behaviour or mortality in the presence of actual predators, our research largely supports the hypothesis that prey naivety may be an important factor in population impacts of non-native predators.

To be useful in a management context, these results should be considered along with other factors thought to influence the outcome of novel predator invasions: these include degree of taxonomic similarity between native and invaded communities, competitive interactions and existing species diversity in the invaded range (Sih *et al.*, 2010). Smallmouth bass possess several advantages in being an opportunistic predator that is widely spread by humans (Carey *et al.*, 2011). Evidence also suggests that smallmouth bass can outcompete and displace many native predators (Fritts & Pearsons, 2004; Johnson *et al.*, 2008), a trend which may be augmented in the Pacific Northwest by historically low-diversity fish communities that contained no centrarchids. In this particular management scenario, prey naivety is likely to give smallmouth bass an additional competitive edge, to the detriment of native communities and salmon populations. Even more troubling, is that (as elsewhere) smallmouth bass are only one of a suite of novel non-native predators found in the Pacific Northwest (Sanderson *et al.*, 2009). Further, our results suggest that management priorities for salmon conservation which seek to reduce pikeminnow population densities (i.e. state-funded bounty programme and targeted removals) while favoring smallmouth bass (i.e. catch limits) may be misguided.

Despite the need to improve understanding of how novel predators impact native fish communities, we nevertheless recommend that these results be interpreted cautiously and used as a basis for future work. Logistical constraints in our field location resulted in small sample sizes, particularly in the number of control sites. In addition, our research does not take into account how behavioural responses of wild fish may shift with additional cues available to prey through predator diet, or chemical and social learning; however, we consider this work to be a foundation from which to pursue these questions.

Although laboratory and field results were similar, the difference in effect in the two environments is enlightening. Laboratory results suggested flight or escape as a uniformly prominent response to chemical cues of northern pikeminnow, but this response was only one of several antipredator responses witnessed in the field. Our field study indicates that behavioural responses by individuals ranged from abandonment of feeding territories, reductions in movement (including foraging attempts) and increasing time near the substratum. The timing and extent of these responses further varied from less than a minute to the full 10 mins following introduction of the predator cue. This variation is consistent with several factors which could not be controlled in the field, such as hunger, habitat, cue concentration and group membership: all of these factors have been demonstrated to mediate the strength of antipredator responses (Ferrari *et al.*, 2010). However, we suggest our laboratory and field comparison also strongly reflects context dependent differences in behavioural decision-making by individuals in confined laboratory spaces, by groups (field sites) and by individuals within groups. Unravelling the interactions between biotic, abiotic and community factors which mediate behavioural decision making may be critical to scaling our understanding of prey naivety to the population or landscape level. In a recent presentation of a theoretical framework of naivety in ecosystems, Sih *et al*. (2010) identify integration of behavioural, sensory and invasion ecology as a key area for research. From the field of chemical ecology, Ferrari *et al.* (2010) specifically call for field experiments that bring an "ecological realism of natural populations" to tests of predator-prey hypotheses. We argue that by taking an integrated approach our results contribute not only to the question of the existence and adaptive value of innate responses to chemical cues, but offer insight into mechanisms by which prey naivety operates at an ecological scale.

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Figures

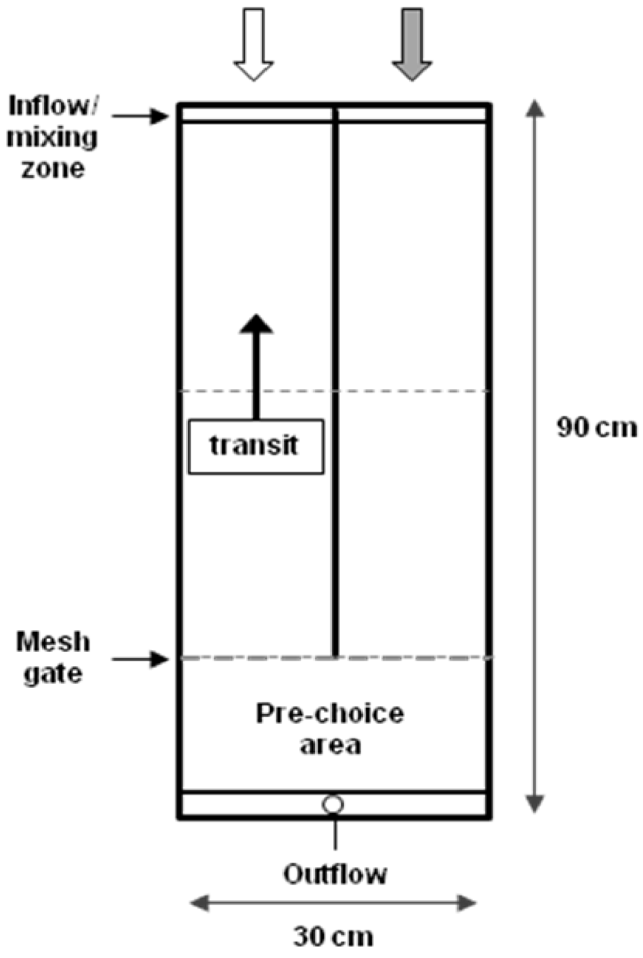

Fig. 1 Schematic of two channel choice flume and grid overlay for video analysis. A 'transit' constituted moving from one section (delineated by dashed lines) into another. Time spent in the pre-choice area did not count as time spent in either channel.

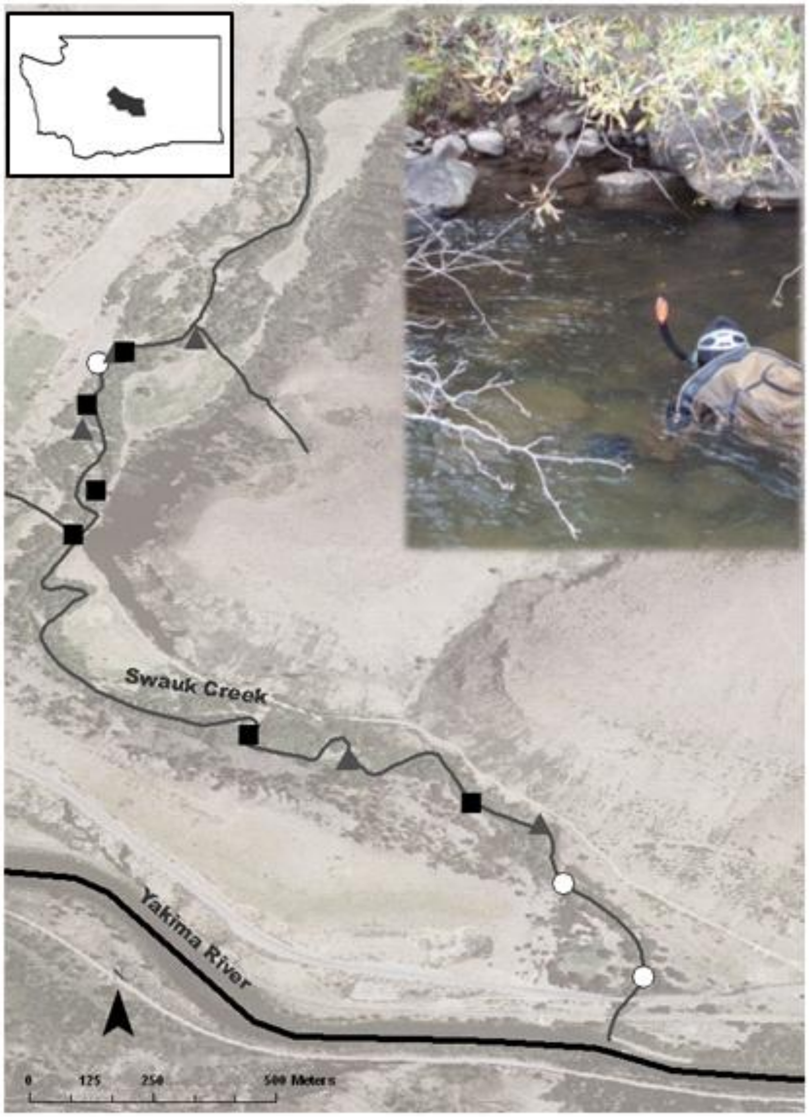

Fig. 2 Experimental sites along Lower Swauk Creek (Kittitas County, Washington State, inset) and example of pool habitats surveyed before and after introduction of predator odour. Treatments are identified as pikeminnow (*P. oregonensis,* black square), smallmouth bass (*M. dolomieu*, grey triangle) and control (well water, open circle).

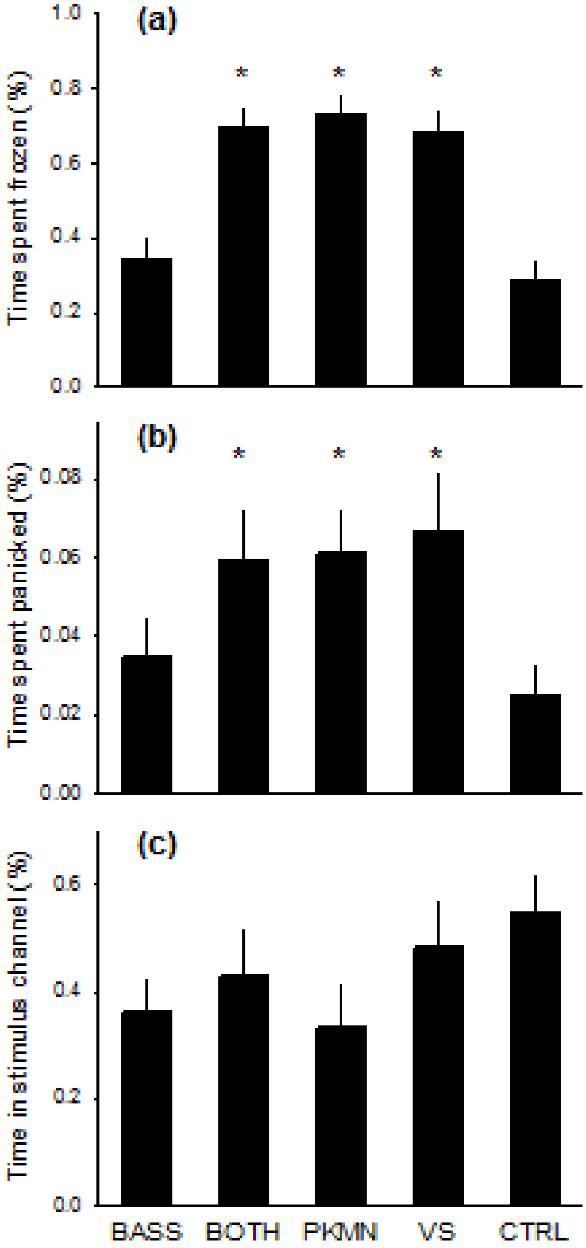

Fig. 3 Percent of time (mean ± SE) spent (a) frozen (b) panicked and (c) in stimulus channel by treatment, and significant *post-hoc* comparisons for 10 min trials. Treatments are bass:control (BASS), bass+pikeminnow:control (BOTH), pikeminnow:control (PKMN), bass:pikeminnow (VS) and control:control (CTRL). *Treatments that are significantly different from the control (Dunnett's test, alpha=0.05).

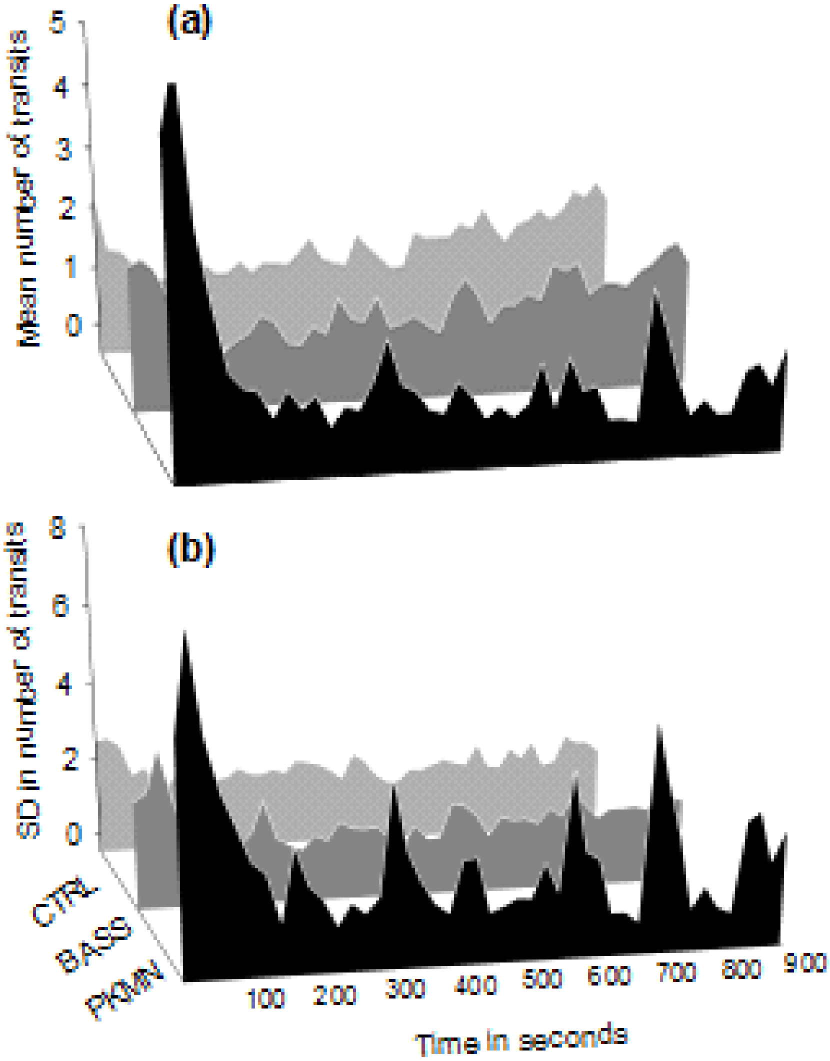

Fig. 4 (a) Mean number and (b) standard deviation of transits by treatment in 15 min trials following gate raise. Treatments are pikeminnow (PKMN), smallmouth bass (BASS) and control (CTRL).

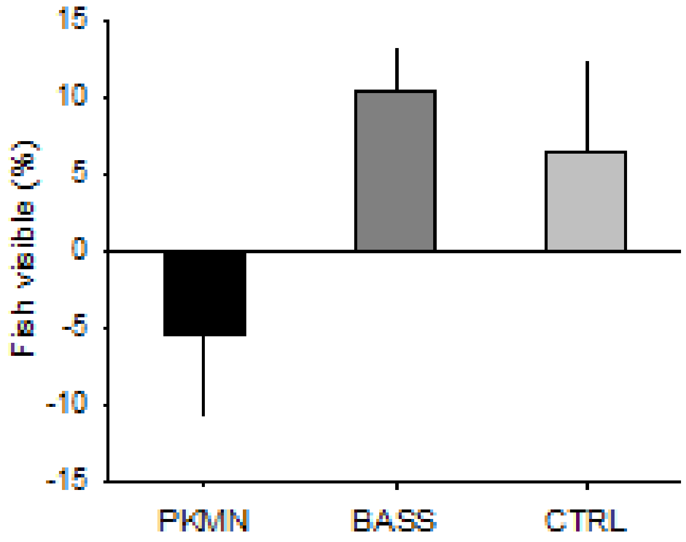

Fig. 5 Change (mean ± SE) in percent of fish visible to snorkeler by treatment for sites exposed to odour of pikeminnow (*n*=6), smallmouth bass (*n*=5) and control (*n*=3). Treatments are pikeminnow (PKMN), smallmouth bass (BASS) and control (CTRL).

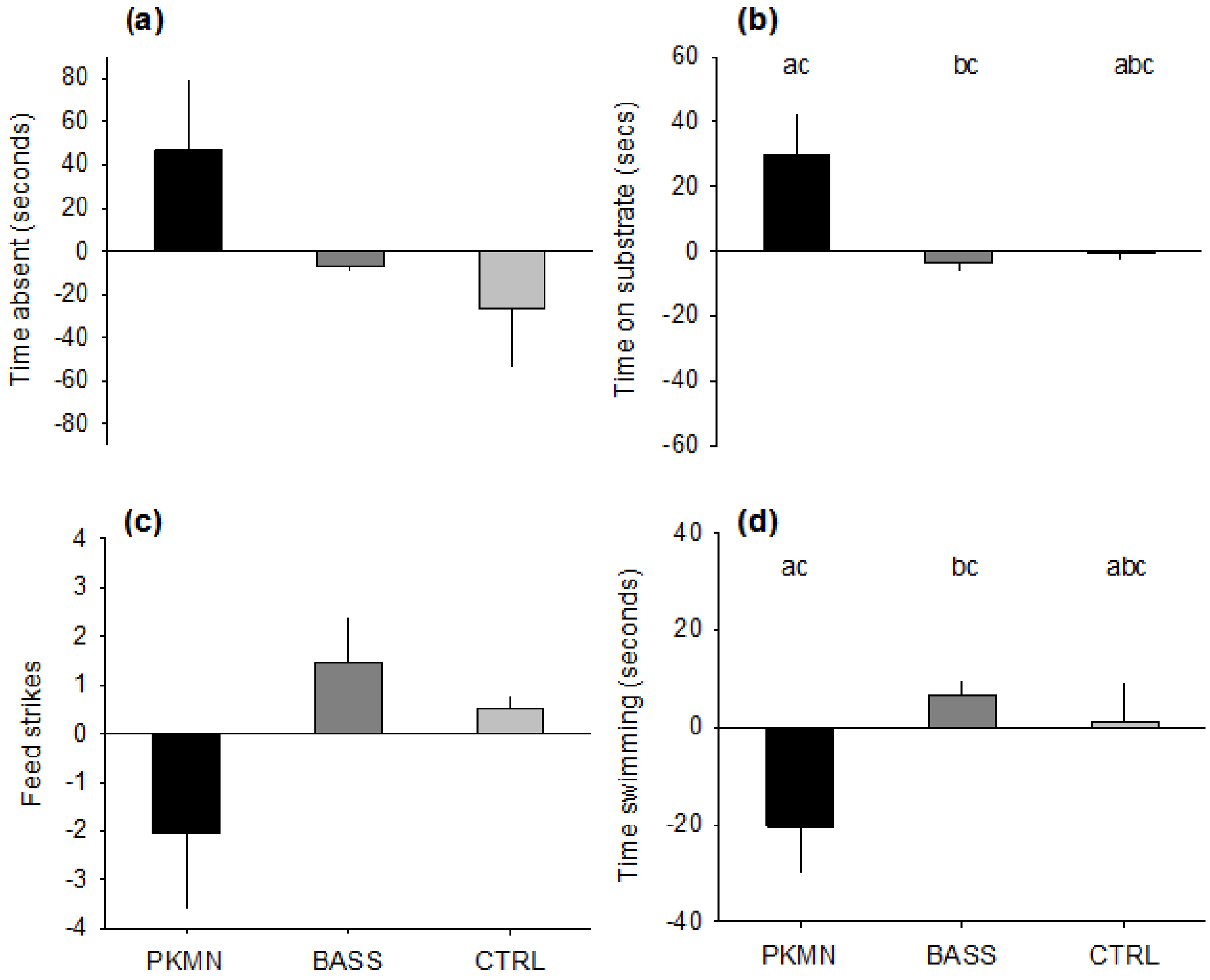

Fig. 6. Change (mean ± SE) in focal follow variables: (a) time spent absent (seconds), b) time spent on substratum (seconds), (c) feeding strikes and (d) time spent swimming (seconds) after introduction of predator odour. Sites were exposed to the odour of pikeminnow (*n*=5), smallmouth bass (*n*=5) and control (*n*=3). Treatments are pikeminnow (PKMN), smallmouth bass (BASS) and control (CTRL). Significant treatment differences are indicated by letter groupings above bars (Tukey-Kramer test, alpha=0.05).

Chapter II: Costs of living for juvenile Chinook salmon in an increasingly warming and invaded world

Abstract

Rapid environmental change has created a need to understand the interactive effects among multiple stressors. In freshwater ecosystems, past and projected changes in temperature and flow regimes and the effects of invasive predators have been identified as key threats to imperiled species. Although mortality is the primary focus in many studies of multiple stressors, sublethal effects can be at least as important in driving the behavior and growth of individuals, leading to effects on population structure and abundance. To examine the separate and interactive effects of multiple stressors on integrated responses, we tested the effects of increased water temperature and predation by non-native smallmouth bass (*Micropterus dolomieu*) on the sublethal (behavior, physiology, and growth) and lethal (mortality) effects for juvenile Chinook salmon (*Oncorhynchus tshawytscha*) in large, semi-natural stream channels over 48-hour trials.

Contrary to expectation, there was no significant difference in mortality of juvenile salmon due to predation in warmer water. Fish in both predator treatments significantly reduced swimming activity and increased surface water shoaling (an antipredator behavior); however, warmer temperatures resulted in stronger and overall more variable behavioral responses to predation. There was no additive effect of temperature and predation on physiological indicators (plasma glucose, plasma cortisol); our results instead show an antagonistic interaction, indicating suppression of physiological mechanisms by the combined stressors. Growth was significantly reduced in the combined treatment, indicating a negative interactive effect of increased temperature and predation.

These results suggest that chronic increases in temperature may not increase vulnerability of juvenile salmon and other freshwater fish to predation over short periods, but can reduce growth and tax physiological and behavioral compensation systems. Long term implications for freshwater fish are reduced ability or consistency in meeting developmental benchmarks necessary for overwinter survival, migration, reproduction, or disease resistance. Our study also indicates that conclusions regarding impacts of multiple stressors will depend on the response variables and time scale on which they are measured. Studies of multiple stressors may benefit from approaches which account for sublethal costs and compensations across different types of organism response.

Introduction

Animals can compensate for stress as a natural part of life; however, large-scale anthropogenic processes have caused unprecedented rates and magnitudes of environmental change (Ellis, 2011). Concerns regarding these changes have led to a demand for investigation into the effects of multiple drivers of ecological change in habitat, climate, pollution, and invasive species on ecosystems (Sih, Bell & Kerby, 2004; Didham *et al.*, 2007; Crain, Kroeker & Halpern, 2008). While there is currently little consensus whether these effects (neutral, synergistic, or antagonistic) can be reasonably predicted (Christensen *et al.*, 2006; Darling & Côté, 2008), there is a pressing need for research that bridges the (generally dire) theoretical and landscape scale predictions of species persistence with understanding of behavioral and physiological capacities to respond to environmental change (Helmuth, Kingsolver & Carrington, 2005; Thrush *et al.*, 2009). Not surprisingly, the potential for synergistic effects of climate change with other stressors is perhaps the primary impetus for integrated research (Brook, Sodhi & Bradshaw, 2008; Fuller *et al.*, 2010). Recent inquiry has also illuminated a particular dearth of research on impacts of multiple stressors in freshwater systems (Strayer, 2010); this is troubling as freshwater organisms may be especially vulnerable due to natural spatial heterogeneity and barriers to dispersal, exacerbated by extensive habitat alteration.

Freshwater fish are ectotherms controlling body temperature through external means, so the effects of changing water temperatures are critical to organism growth and performance (McCullough *et al.*, 2009). In both lentic and lotic systems, loss of riparian vegetation elevates water temperatures (Allan, 2004), dams modify downstream thermal regimes (Olden & Naiman, 2010), and both past trends and climate change models indicate warmer water temperatures for many regions (Nelson *et al.*, 2009; Kaushal *et al.*, 2010). Prior work on the effects of temperature on freshwater fish has emphasized lethal limits (Lutterschmidt & Hutchison, 1997) and acute changes associated with hydropower systems (Schreck, 2000), leaving the impacts of chronic temperature stress on growth, development, and disease resistance less well studied (but see Morgan, McDonald & Wood, 2001; Marine & Cech, Jr., 2004).

Coincidental with temperature, predation also affects fish population dynamics; however, the extent to which temperature may mediate predator-prey interactions has rarely been tested experimentally. This represents an important knowledge gap for two reasons. Species ranges and distributions have already responded to past climate change by showing shifts to higher latitudes and elevations (Hickling *et al.*, 2006); this is expected to change the spatiotemporal overlap of many predators and their prey. Invasions or introductions of warm-water species into new systems and habitats are also occurring, creating novel predator-prey encounters (Rahel & Olden, 2008) which may result in greater predation pressure than by native predators (Salo *et al.*, 2007). In addition, changes in temperature cues can shift the timing of life history events such as emergence or migration (Parmesan & Yohe, 2003), but these shifts are not uniform across species in a community (Both *et al.*, 2009).

In this study, we tested the effects of increased water temperature on the vulnerability of juvenile Chinook (*Oncorhynchus tshawytscha*) salmon to both direct mortality and sublethal (behavior, physiology, and growth) effects of predation by non-native smallmouth bass (*Micropterus dolomieu*) using large, semi-natural stream channels. Our choice of prey and predator reflects significant management interest in the impacts of warm-water invaders in freshwater ecosystems (Vander Zanden & Olden, 2008), and more specifically the impacts of non-native predators on threatened Pacific salmon as cold-water stenotherms highly sensitive to climate-induced warming (Schindler *et al.*, 2008; Sanderson, Barnas & Rub, 2009). Smallmouth bass were initially transplanted from eastern North America to the Pacific Northwest in the 1920's; subsequent decades saw continued stocking by state agencies and private citizens, with establishment of self-sustaining populations in lake and river systems. Smallmouth bass have become an increasingly conspicuous predator of juvenile Pacific salmon over the last two decades (Carey *et al.*, 2011). Although estimated predation rates vary across the region, analysis over several outmigration periods (March – June) suggests that smallmouth bass predation could account for between 4-35% of wild fall-run Chinook smolts in the lower Yakima River, depending on environmental conditions (Fritts & Pearsons, 2004). The interactive effects of temperature and predation are potentially of great importance to the viability of this culturally and commercially important family of species.

Our study objectives were to test *a)* the hypothesis that warmer temperatures would result in increased rates of predation by smallmouth bass and *b)* the hypothesis that temperature and predation would result in different interactive effects – neutral, synergistic, or antagonistic in manifestation – on different types of organism response. We intended to address a knowledge gap by integrating responses to multiple stressors across behavior, physiology, growth and mortality in an ecologically realistic setting (Helmuth *et al.*, 2005). In the past, the focus on response to multiple stressors has largely been on mortality (Darling & Côté, 2008); although understandable, this does not take into account that animals accept significant sublethal costs (e.g. growth, optimal habitat or mating opportunities) to avoid mortality (Werner & Peacor, 2003). The 'risk-sensitive hypothesis' has been well studied in predator-prey interactions, demonstrating that animals behave in ways that prioritize and balance costs; this prioritization process can drive patterns of animal distribution, growth, and survival at landscape scales (Preisser, Bolnick & Benard, 2005; Biro *et al.*, 2006; Heithaus *et al.*, 2008). By taking an integrated approach we can identify whether different kinds of stressors (e.g. biotic or abiotic) exert pressure on different or similar aspects of biological response, and also examine how individuals prioritize and respond to multiple threats.

In this manuscript, we have used the terms 'lethal effect' to describe mortality due to predation and 'sublethal effects' for behavior and physiological responses (including growth). Consumptive and nonconsumptive effects (CE and NCE) have also been used to describe these kinds of responses to predators; lethal and sublethal are clearer for our purposes as our experimental factors include temperature as well as predation, but our results are easily translated to CE and NCE concepts.

Methods

Test fish

Spring-run (Yakima River population) Chinook salmon eggs were acquired by the Cle Elum Supplementation Research Facility from 15 families of natural origin (wild broodstock) spawned during September 2009. The eggs were transferred to the Northwest Fisheries Science Center (National Oceanic and Atmospheric Administration), and incubated at 5°C until they began feeding in mid-February 2010. In early April, the fish (approximate fork length 40 mm) were transported to the Western Fisheries Research Center (WFRC; United States Geological Survey), and reared in sand-filtered and UV-treated freshwater from Lake Washington at ambient intake temperatures (mean ± SD = 10.7°C ± 1.0°C) in a 700-L circular tank. Juvenile Chinook salmon were held on a natural photoperiod regime and fed twice daily *ad libitum* (Bio Oregon bioVita) until trials began in early August, when fish measured 85.0 ± 7.0 mm (fork length: mean ± SD).

Fifteen smallmouth bass predators (fork length: mean ± SD = 362 ± 23 mm) were captured by angling from Lake Washington, and housed in an unused stream channel at ambient temperatures (mean ± SD = 14.8 ± 1.4°C) until the trials began. This allowed them to habituate in a very similar environment to the experimental channels for 6-8 weeks prior to trials. Predators were maintained on a mixed diet (earthworms, crayfish, Chinook salmon), and were only used in trials if they regularly consumed live prey. The length ratio of prey to predator did not exceed 26% of any individual predator, well within the 50% maximum reported for smallmouth bass preying on salmonids in the wild (Fritts & Pearsons, 2006). Preliminary trials also confirmed that smallmouth bass were effective and efficient predators on juvenile Chinook salmon in this size range (*L. Kuehne, unpublished data*).

Experimental arenas

Trials took place in four large experimental channels (1.2 m *w* x 5 m *l* x 1 m *d*) under a covered outdoor pavilion on the WFRC campus, which conveyed partial light through skylights (Figure 1). The water was supplied from nearby Lake Washington through sand-and UV-filters, and circulated continuously by pumps at 0.1 m sec^{-1} within the channels. Channel temperatures were set at 15°C (hereafter 'cool') and 20°C (hereafter 'warm'); these temperatures were selected as representing the normal and upper range of mid-summer temperatures encountered in juvenile Chinook rearing areas (Beckman *et al.*, 2000; Quigley & Hinch, 2006), and which are expected to occur with greater frequency and duration under regional climate change scenarios (Mote et al. 2003).

Within channels, a mixture of gravel and cobble was landscaped into upper (0.8 m *d*), middle (1 m *d*), and lower (0.8 m *d*) sections (ordered downstream); each section contained two groups of large cobble affording some refuge from predators without concealing fish from the observer. Channel interiors were viewable through six regularly spaced 25 x 22 cm acrylic windows. An underwater camera (Speco Technologies, Model CVC321WP) was mounted at the head of each channel to record predator activity for subsequent analysis (Figure 1). A removable seine net was positioned 2/3 of the way down each channel to create separate acclimation areas for predators and prey.

The five consecutive trials were conducted between 7-27 August, 2010. A trial consisted of four separate treatments: (1) cool, (2) cool + predator, (3) warm, and (4) warm + predator. Temperature and light levels in each channel were recorded every 2 h during trials (Onset Corp, UA-002-08). Although incoming lake temperatures were subject to some variation, temperatures between trials were highly consistent (cool mean ± SD: 14.9 ± 0.7°C, warm mean ± SD: 20.2 ±

0.8°C). Because channel temperatures could not be changed easily, warm and cool channels remained constant between trials, but predator treatments were randomized.

Behavior and physiology data collection

Juvenile Chinook salmon were acclimated to cool and warm treatment temperatures over 7 days at rates of 0.5 and 1.5 °C day^{-1}, respectively, to avoid stress responses due to heat shock (G. Sanders, USGS Veterinary Officer, personal communication). At the start of each trial (07:00 h), 20 salmon were transferred to each experimental channel and allowed to acclimate for 12 h ('acclimation period') before predators were introduced behind block nets. Preliminary trials indicated that juvenile salmon took approximately 16-24 h to establish feeding territories, consistent with other stream channel studies (Taylor, 1988). Initial length and weight estimates were obtained from 10 additional salmon (5 cool and 5 warm) acclimated for the purpose of establishing initial size without introducing handling stress by anesthetizing and measuring all fish. At 19:00 h, smallmouth bass were transferred to the experimental channels; bass and salmon acclimated in their separate areas overnight, and block nets were removed at 07:00 h the following morning. Predators and prey then freely accessed the entire channel for 48 h ('predation period').

During predation periods, standardized behavioral surveys of Chinook salmon were conducted every three hours between 07:30 and 19:30 h. Three individual focal fish were selected randomly starting in the lower, middle, and upper sections, ensuring representation throughout the stream channel. For each separate focal follow, we quantified for a one minute period the: 1) number of seconds spent swimming, 2) degree of shoaling based on an index, 3) vertical position in the water column, 4) number of feed strikes, and 5) number of aggressive encounters (Table 1). Vertical position in the water column was scored every 10 seconds by the observer (based on channel wall markings) using an index of (0) bottom 25 cm, (1) middle 25 cm, (2) upper 25 cm, and (3) 5 cm surface layer. Shoaling was also scored every 10 seconds using an index from 1 (further than 2 body lengths from any other fish) to 5 (within 1 body length of two or more fish). Observations were made from a downstream position whenever possible. In general, juvenile salmon appeared relatively undisturbed by an observer; only 3 of 600 focal surveys were abandoned because a fish exhibited abnormal behavior attributed to the observer. Ability of the observer to locate all fish in the channel during surveys was high (mean ± SD: 91 ± 5% of fish per survey).

The natural supply of invertebrate prey (mean ± SD: 0.13 ± 0.07 l^{-1}) consisting primarily of cladocerans, copepods, and chironomids in the channels was supplemented with chironomid larvae delivered every 6 hours, and introduced after surveys to avoid recording acute behavioral changes in response to increased food. Frozen cubes (3 g) were added to a perforated bottle upstream of the screen between the pump and experimental area. Water flowing through the bottle gradually carried chironomids into the channel; although exit times were variable, typical dispersion occurred within 60 minutes. This amounted to an approximate daily food ration of 5% body weight delivered to each channel; although this food was unlikely to be evenly obtained by individuals, it provided a standardized ration across trials and treatments in addition to naturally occurring invertebrate prey.

At the end of trials, all fish were removed from channels and blood samples collected from salmon for analysis of plasma cortisol and plasma glucose. These indicators, well-studied in salmonids, were chosen for the potential to offer insight into primary and secondary physiological stress response (Barton, 2002). Smallmouth bass were quickly netted; surviving salmon prey were removed by electro-fishing (Smith-Root, Model L-24) and placed directly in 220 mg l^{-1} tricaine (MS-222). Length measurements were taken for all fish, and blood collected with a heparinized Natelson capillary tube at the severed caudal peduncle. Removal of fish and sampling were done rapidly to avoid acute increases in plasma cortisol; time to obtain samples after initiating electro-fishing was 4-6 minutes. Blood samples were centrifuged for 10 minutes at 5°C, plasma extracted and stored at -20°C until analysis. Plasma glucose levels ($n=7$ for each trial and treatment) were measured in duplicate using the hexokinase method available in kit form (Stanbio Laboratory, Boerne, TX). Samples were read at 340 nm on a spectrophotometer (Thermo Electron Corporation, Madison, WI), and intra- and inter-assay coefficients of variation were less than 5%. Plasma cortisol concentration ($n=5$ for each trial and treatment) was measured using a radioimmunoassay (Siemens Diagnostics, Los Angeles, CA). Samples were twice ether-extracted (extraction efficiency 75%), and extracts were dried and reconstituted in the same matrix as the standards. Two additional standards of 2.5 ng ml^{-1} and 5 ng ml^{-1} were added to the standard curve; these standards bound at 94% and 88% respectively, and were within the reported assay sensitivity of 2 ng ml^{-1}. All samples were assayed together, with a CV of 3.82%. Samples were assayed at the Washington State University Center for Reproductive Biology Assay Core Lab.

Growth and mortality data collection

The fork length (FL) of all surviving fish was measured, and body weight assigned using a regression of final lengths and weights of at least 5 fish from each treatment ($R^2=0.92$, $p < 0.01$, $n=112$). These body weight data were bootstrapped 100 times (5 fish selected without replacement) to estimate a mean final weight for each trial and treatment. A specific growth rate (SGR) was then calculated using the initial mean weight of the fish subsampled at the start of each trial. SGR (% body weight day^{-1}) was calculated as $SGR = (\log_n wt_2 - \log_n \mathrm{wt}_1)/\mathrm{time}$, where wt_2 = final weight (mean of 5 fish, bootstrapped) and wt_1 = initial weight (mean of 5 fish, subsample). The time interval was 3 days, which included stream channel acclimation and predation periods.

Direct predation of juvenile Chinook salmon by smallmouth bass was inferred from the number of survivors remaining in channels, and smallmouth bass activity was later quantified using video recordings from the underwater cameras (Figure 1). For the 10 min time period corresponding to behavioral surveys of salmon, an observer recorded the number of 1) transits between upper, middle and lower sections, and 2) attacks on salmon prey by smallmouth bass.

Statistical Analysis

From the 600 one-minute focal follows of juvenile salmon, the following average metrics were calculated for each trial and treatment: (1) percent time swimming, (2) number of foraging attempts, (3) shoaling score, (4) vertical position score, and (5) number of aggressive encounters (Table 1). Given the expectation of some correlation in behavioral responses, ordination using principal components analysis (PCA) was paired with permutational MANOVA (Anderson, 2001; PERMANOVA on standardized data with 9,999 permutations) to visualize relationships between variables and test for an overall effect of treatment on prey behavior. Subsequent PERMANOVA and a test for homogeneity of multivariate dispersions (Anderson, 2006) were conducted between each treatment combination to assess pairwise differences between overall behavior and variability in behavioral response, respectively. Although permutational approaches offer greater freedom from assumptions of normality and heteroscedascity, the behavioral dataset was nonetheless examined for and met assumptions of univariate and multivariate normality.

Mean values of plasma glucose (*n*=7), plasma cortisol (*n*=5), and specific growth rate (*n=5*) were calculated for each trial and treatment. Mortality, predator attacks, and predator activity were summarized by trial and treatment for predator treatments. Behavior scores from principal components 1 and 2 were also extracted for further analysis. For each of these variables, treatment effects relative to the cool reference group were analyzed using separate linear mixed models which included trial as a random effect. Use of the mixed model allowed us to control for variation between experimental blocks (trials) as well as compare the direction and magnitude of treatment effects relative to a reference treatment through analysis of regression coefficients and associated t-statistics (Faraway, 2006; Crawley, 2007). All variables were examined for and met assumptions of normality and heteroscedascity; all statistical analyses were conducted in the R Programming Environment using the vegan and nmle packages.

Results

Effect of temperature and predation on behavior and physiology

Multivariate summary of the behavioral responses revealed differences between temperature, predation, and combined treatment groups, with 66.9% of the variation explained by the first two principal components (Fig. 2). The first principal component (43.5% of variation explained, $p < 0.01$) distinguished increased shoaling activity, increased vertical position (proximity to the surface), and reduced time swimming in predator treatments compared to no-predator treatments. This effect was greater in the warm + predator treatment, indicating that fish in the warmer water increased the strength of these antipredator behaviors. Aggression and feeding were associated with the second principal component, with fish in both predator treatments feeding less and increasing aggression; however, this axis did not explain a significant amount of variation in the behavioral dataset (23.4%, $p = 0.76$). When the behavioral patterns (mean responses in reduced ordination space) were tested for statistical significance between treatments, both predator treatments were significantly different compared to the cool treatment, in addition to the warm versus warm + predator treatment (Table 2). Differences in overall variation of behavioral response between treatments were more minimal, where only the warm + predator treatment showed significantly greater dispersion around the centroid (Table 2, Fig. 2).

The behavioral patterns visualized in the ordination are supported by linear mixed model analysis on the first two principal components (PCI and PCII), which tested for significant differences in dominant behavioral trends against the cool reference treatment. This resulted in a significant treatment effect for PCI only (Table 4a), which primarily described antipredator responses of shoaling, proximity to the surface, and reduced time swimming. The significant difference in the warm + predator treatment (regression coefficient = 2.28, $t_{12} = 2.99$; $p = 0.01$) reflects the greater response in these behaviors relative to the cool reference treatment. Analysis of PC2 (aggression and feeding) resulted in no significant differences between reference and other treatments (Table 4a; $t_{12} < 1.07$, $p > 0.30$).

Juvenile salmon exhibited elevated plasma glucose in both warm temperature and predator treatments compared to the cool reference treatment, but differed in magnitude and significance of response (Table 3, Table 4a). The largest and statistically significant increase occurred in the cool + predator treatment (regression coefficient = 9.6, $t_{12} = 2.87$; $p = 0.01$), whereas glucose was similarly, but less, elevated in warm and warm + predator treatments (regression coefficients = 6.4 and 5.4, $t_{12} < 1.92$; $p > 0.08$). Plasma cortisol levels showed similar patterns to plasma glucose relative to the reference treatment in mixed model analysis (Table 3, Table 4a). Plasma cortisol increased significantly in both predator treatments (regression coefficients > 23.9, $t_{12} > 2.24$; $p < 0.045$), with the largest increase in the cool + predator treatment.

Effect of temperature and predation on growth and mortality

Short-term trends in growth of juvenile salmon were positive in the cool reference treatment, trending toward neutral and negative growth in predation, temperature, and combined treatments (Table 3). Relative to the cool reference treatment, the largest and statistically significant reduction in growth occurred in the warm + predator treatment (Table 4a; regression coefficient=-6.1, $t_{12}=2.39$; $p=0.03$), indicating that fish grew the slowest in response to the combined stressors.

Direct predation from smallmouth bass was generally low, with a mean of 11% (range: 0-25%) across all trials, equating to an average consumption rate of 1.15 juvenile Chinook salmon predator^{-1}day^{1} (Table 3). Warmer temperatures did not significantly affect predation of salmon (Table 4b; regression coefficient =1.0, $t_5 < 0.27$; $p = 0.80$), nor did increased temperatures result in any significant differences in bass activity (number of attacks witnessed or transits observed during behavioral surveys; Table 4b; regression coefficients = -0.1 and 0.6, $t_5 < 0.07$; $p > 0.94$).

Discussion

This study revealed neutral, additive, and synergistic effects of increased temperature and bass predation on juvenile Chinook salmon, but the direction and magnitude of the interaction depended on the response variable. By investigating the effects of multiple stressors across different types of response, our study contributes to an enhanced understanding of how behavior and physiology is related to whole-organism performance at the levels of growth and mortality. Our study is also unusual in measuring these responses in semi-natural and ecologically relevant conditions of sublethal temperature rise and actual predation (as opposed to non-lethal predator cues) under predator-prey densities seen in wild fish populations.

We found no effect of increased temperature on direct predation of juvenile Chinook salmon, contrary to the expectation that predation would increase in the warmer experimental treatment due to increased metabolic demand nearer the thermal preferences (approximately 30°C) for smallmouth bass (Johnson & Kelsch, 1998). Consumption of Chinook salmon averaged 1.15 juvenile salmon predator^{-1}day^{-1} over the 48 hour trials. These rates are higher than, but not inconsistent with predation by smallmouth bass in the wild, where predators are also consuming other sources of prey; during summer months reported or estimated consumption rates are 0.02 – 0.50 salmon predator^{-1}day^{-1} (Rieman *et al.*, 1991; Tabor, Shively & Poe, 1993; Tabor *et al.*, 2007). However, our experimental predation rates were considerably lower than in preliminary trials where smallmouth bass were (presumably) less habituated to laboratory feeding and were also not solitary (*L. Kuehne, unpublished data*). Although convenient for experimental purposes by reducing potentially large variability in survivorship and sublethal effects due to differences between individual predators, this suggests the experimental design was perhaps more ecologically relevant to salmon prey than to smallmouth bass, which were likely to experience stress from solitude and trials that were relatively short in duration. In more realistic predation scenarios (e.g., multiple predators in natural environments over longer time periods), thermal-metabolic predictions of increased consumption with warmer temperatures might be manifested.

Our results are consistent with other studies examining the ability of juvenile salmon to compensate for short-to-medium term stressors in terms of vulnerability to predation, although these studies were conducted largely within the context of acute stressors experienced during outmigration through hydropower systems. Mesa (1994) and Mesa, Weiland & Wagner (2002) examined effects of acute temperature increases and sequential handling events on predator avoidance ability and physiology of juvenile Chinook salmon. Both studies, conducted over similar periods as our experiment (24-48 hours), found no difference (or rapid recovery) in predator avoidance ability, although significant changes in physiological responses occurred. Even given differences in experimental design, we believe our results lie within this body of work to support a model of short-term compensation to stressors which avoids increased vulnerability to predation (Barton, 2002). However, long-term responses to stress include reduced growth and development, and delayed mortality of aquatic organisms (Marine & Cech, Jr., 2004; McCauley, Rowe & Fortin, 2011), and therefore should be considered.

Our salmon prey exhibited appropriate behavioral responses to predators (regardless of temperature) by increasing shoaling, shifting to a higher vertical position, reducing activity, and reducing foraging. However, these behaviors differed significantly from reference conditions only when predation was combined with warmer temperatures. The few studies which have investigated temperature-driven behavior of fishes indicate increased activity rates, to some extent attributed to increased foraging effort (Claireaux *et al.*, 1995; Quigley & Hinch, 2006;

Biro, Beckmann & Stamps, 2009). Although we also saw slight increases in activity in response to elevated temperature in isolation, when combined with predation this resulted in an opposing trend of stronger (and overall more variable) antipredator behaviors; interestingly this did not appear to result in an additive reduction in foraging. Interpretation of behavioral responses to stress is made difficult by the fact that animal behavior is often the result of competing motivations which are poorly understood (Rushen, 2000); this may be particularly true of our study in semi-natural conditions where ‘motivation’ in terms of predator threat, natural food supply, and initial size of juvenile salmon varied over time and across trials. However, the additive effect of temperature and predation on behavioral responses is notable, and suggests an attempt to compensate for increased vulnerability to predation at some level or to offset the additive growth costs of the combined stressors.

As with behavior, trends in primary (plasma cortisol) and secondary (plasma glucose) physiological stress indicators were reasonable and consistent with other studies. Although cortisol is notoriously difficult to sample in semi-natural and natural conditions due to rapid response time, the range of plasma cortisol values we report are similar to those documented for this same species within 30 minutes of a single disturbance event (i.e. movement of holding tank) (Mesa, 1994). This suggests that our rapid sampling largely avoided extreme cortisol elevations that can occur in response to sampling procedures, and that the fish in our study were exhibiting moderate as opposed to acute stress responses to increased temperature and predation. Fish in both predator treatments had elevated plasma cortisol, but with no evidence of additive effects of the two stressors. This pattern is mirrored in the response of plasma glucose, which was significantly elevated only in the cool + predator treatment. As a secondary stress response, plasma glucose is less likely to be influenced by sampling procedures when conducted rapidly, and our values are consistent with those reported by Mesa (1994) in response to the single disturbance event. Based on studies examining physiological responses to acute stressors, we expected a possible additive effect (Barton, Schreck & Sigismondi, 1986; Mesa, Weiland & Wagner, 2002; Quigley & Hinch, 2006). Our failure to find this in the context of moderate or chronic stressors, and to see evidence instead for an antagonistic or diminished effect is, however, not without support. Although metabolic mechanisms and feedbacks are complex, strong evidence indicates that chronically stressful conditions can down-regulate the hypothalamic-pituitary-interrenal (HPI) axis which is responsible for cortisol response, reducing response to subsequent stressors (Barton, 2002). Plasma glucose (and other secondary stress responses) have also exhibited reduced response under conditions of chronic or sustained stress (Barton, Schreck & Barton, 1987; Mesa *et al.*, 1998; Schreck, 2000). In our study, increased temperature represents a sustained stressor while predation conceivably occurs as a series of acute events (i.e., when predators are actively hunting); taken together the trends in our physiological data suggest that temperature and predation, in combination, can result in suppressed physiological responses to stress over relatively short periods.

Lastly, our results showed an additive effect of multiple stressors on the growth of juvenile Chinook salmon, evidenced by a significant negative effect in the warm + predator treatment. We have also documented significant behavioral and physiological responses corresponding to these patterns in growth, such as reduced feeding in predator treatments coupled with a) more conservative antipredator behaviors and b) increased metabolic demands in the warmer treatment. The specific mechanisms of behavioral and physiological coupling which mediate growth in different contexts (Stoks *et al.*, 2005; Steiner, 2007), while very important to understand, are beyond the scope of this work: our intention was to investigate impacts of multiple stressors at an ecologically meaningful scale which still allowed careful measurement of biological response. Our ability to estimate growth within this experimental design had the potential to be confounded by size-selective predation by smallmouth bass, which has been documented in the wild (Fritts & Pearsons, 2004). However, not only were predation rates low (in some trials equal to zero), but if size-selection was occurring and the smallest individuals were removed by predators, our estimates of reduced growth (based on a final group mean) would be conservative. Further, although our growth estimates may be subject to subtle size biases which are often inevitable in group sampling, the systematic nature of these biases gives us confidence in the relative trends between treatments. Given that individual size and growth rate is often the best predictor of subsequent survival and reproductive success of juvenile salmonids (Zabel & Achord, 2004; Reed, Martinek & Quinn, 2010; Duffy & Beauchamp, 2011), the additive negative impact of temperature and predation indicates the strong incentives that these (and other) animals have to avoid suboptimal habitats resulting in reduced growth potential.

Schreck (2000) reviewed the cumulative and long-term effects of stress in fish and noted the lack of empirical knowledge on multiple stressors. He proposed that fish living near the tolerance limit of one stressor will exhibit magnified responses to a second stressor, but that these will be primarily manifested at the level of performance (e.g. behavior, development, or reproduction). Our results are consistent with this prediction; at temperatures near the upper range of thermal tolerance for juvenile Chinook salmon, we saw magnified behavioral responses to a second stressor (predation), and magnified costs in growth. Our results do not indicate that these costs extended to increased mortality due to predation in the short term, but it is likely that over longer periods fish exposed to stressful environmental conditions such as these would be more likely to succumb to size-selective predation, increased vulnerability to disease, or impaired development (Schreck, 2000; Marine & Cech, Jr., 2004; Cairns *et al.*, 2005).

We believe that our study results, which integrate multiple responses of juvenile Chinook salmon to two ubiquitous stressors, demonstrate the relative importance of lethal and sublethal effects to species undergoing rapid environmental change. Focus in conservation biology is often on direct mortality or decline in population abundance due to acute stressors or biotic interactions; however, the insidious (and challenging to quantify) effects of cumulative stressors at the level of behavior, physiology, and development may be of equal or greater importance in the long-term viability of populations. We suggest that integrated studies will result in more accurate estimates of consequences of multiple stressors on whole organism performance, including animal behavior and distribution at landscape scales.

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Tables

Table 1. Behavioral metrics assessed for juvenile Chinook salmon (real-time) and smallmouth bass predators (analysis of video footage) every 3 hours during trials. Ordinal metrics for juvenile salmon prey were characterized every 10 seconds during one-minute focal follows.

Species ***Variable***	**Description**
Chinook salmon	
Time Swimming	Time in seconds of continuous and directional movement resulting in net displacement
Vertical Position	Score of vertical position in water column using index of (0) bottom 25 cm, (1) middle 25 cm, (2) top 25 cm, and (3) surface 5 cm.
Feed Strikes	Biting either in the water column or on the substrate
Aggression	Nips, charges, or chases
Shoaling Index	Score using index of (1) further than 2 body lengths from any other individual fish to (5) within 1 body length of two or more individuals
Smallmouth bass	
Transits	Number of sections in channel (upper, middle, low) visited each minute
Attacks	Attacks on salmon prey

Table 2. Results of pairwise permutational MANOVA (pseudo P-values above diagonal) and tests of multivariate dispersion (permuted P-values below diagonal) between treatment groups. Significant ($p < 0.05$) differences are in bold.

Treatment	**C**	**CP**	**W**	**WP**
C	-	**< 0.01**	0.76	**0.04**
CP	0.61	-	0.15	0.43
W	0.15	0.50	-	**0.04**
WP	**0.04**	0.11	0.25	-

Table 3. Values (mean ± SD) of selected physiology, growth, and consumption metrics in the four treatment groups: Cool (C), Warm (W), Cool+Predator (CP), and Warm+Predator (WP).

	C	**CP**	**W**	**WP**
Plasma glucose (mg dl^{-1})	76.9 ± 5.4	86.5 ± 4.9	83.4 ± 7.3	82.3 ± 4.8
Plasma cortisol (ng ml^{-1})	57.3 ± 36.4	86.9 ± 35.0	67.9 ± 24.0	81.2 ± 45.1
Specific growth rate (% day^{-1})	2.2 ± 3.8	-0.7 ± 5.1	-0.2 ± 5.5	-4.0 ± 2.2
Consumption (predator $^{-1}$ day^{-1})	n/a	1.15 ± 0.9	n/a	1.20 ± 1.0

Table 4. Results of linear mixed models for treatment effect on response variables; models included trial as a random effect to control for variation between experimental blocks. (A) Treatment effect on behavior, physiology, and growth metrics relative to the “cool” reference group. (B) The effect of temperature on predation and predator activity relative to the “cool + predator” treatment. Direction and strength of the effect is indicated by the regression coefficient; significant ($p < 0.05$) effects are highlighted in bold.

A. All Treatments	**Cool + Predator**	**Warm**	**Warm + Predator**
Variable		regression coefficient	
units		*t value, (p)*	
Behavior PCI	+ 0.88	- 0.05	**+ 2.29**
n/a	*1.15, (0.27)*	*0.06, (0.95)*	*2.99, (0.01)*
Behavior PCII	- 0.70	+ 0.57	- 0.61
n/a	*1.07, (0.30)*	*0.88, (0.40)*	*0.93, (0.37)*
Glucose	**+ 9.6**	+ 6.4	+ 5.4
$mg\ dl^{-1}$	*2.87, (0.01)*	*1.92, (0.08)*	*1.62, (0.13)*
Cortisol	**+ 29.6**	+ 10.6	**+ 23.9**
$ng\ ml^{-1}$	*2.77, (0.02)*	*0.99, (0.34)*	*2.24, (0.045)*
Specific growth rate	- 2.9	- 2.4	**- 6.1**
$\%\ day^{-1}$	*1.12, (0.28)*	*0.92, (0.38)*	*2.39, (0.03)*

B. Predator Treatments	**Warm + Predator**
Mortality	+ 1.0
Percent	*0.27, (0.80)*
Attacks	- 0.1
Sum	*0.07, (0.94)*
Predator Transits	+ 0.6
Sum	*0.06, (0.95)*

Figures

Figure 1. Above water view of stream channels inside of covered pavilion (left panel). Underwater longitudinal view of channel showing smallmouth bass and juvenile Chinook salmon in foreground (top right). Station for rapid blood sampling at end of trials (bottom right).

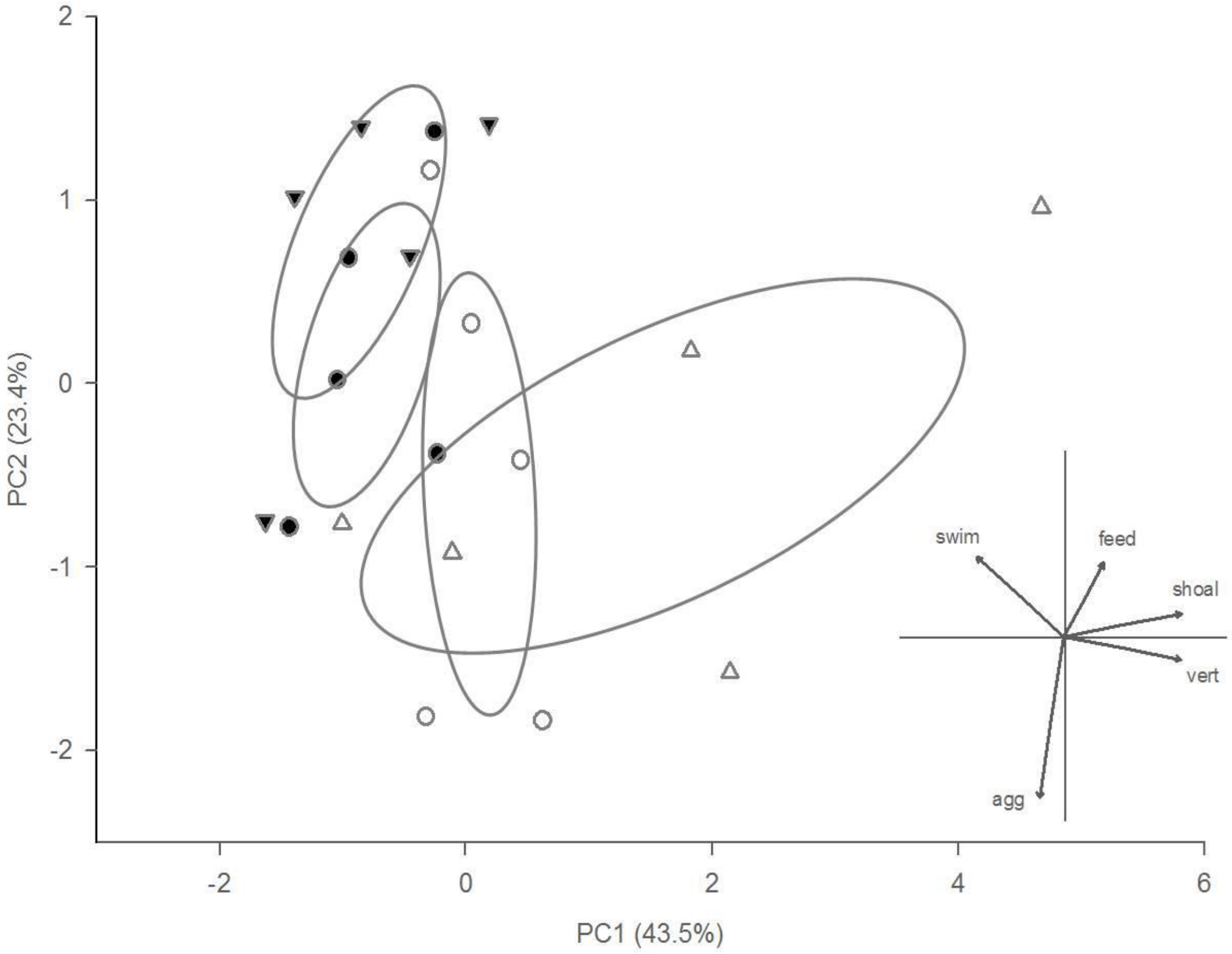

Figure 2. Principal Component Analysis (PCA) of mean behavioral responses for each trial and treatment combination (*n*=5). Treatment groups are delineated with ordination hulls (95% confidence interval) and labeled as Cool (●), Cool+Predator (○), Warm (▼), and Warm+Predator (Δ). The inset indicates the importance of each behavioral metric to the first and second axes, which jointly explain 66.9% of the variation in the behavioral dataset. Behavioral metrics from focal follows of individual fish (Table 1) are coded as: time spent swimming (swim), number of feed attempts (feed), degree of shoaling (shoal), vertical position in the water column (vert), and number of aggressive encounters (agg).