

# Pumpkinseed sunfish (*Lepomis gibbosus*) from littoral and limnetic habitats differ in stress responsiveness independent of environmental complexity and presence of conspecifics

C.B. Belanger, K.S. Peiman, M.N. Vera-Chang, T.W. Moon, and S.J. Cooke

**Abstract:** In the face of a changing world, there has been increasing interest in the behavioural and physiological responses of wild animals to stressors. Many factors can influence stress responsiveness, but two that have not been extensively studied during the stress-induced phase are environmental complexity and the presence of conspecifics. Using wild pumpkinseed sunfish (*Lepomis gibbosus* (L., 1758)) collected from limnetic and littoral sites, we tested whether glucose and cortisol were affected by environmental complexity and the density of conspecifics during the period of maximum response following a standardized air stressor. Overall, environmental complexity and conspecific density did not have a significant effect on maximum stress. However, in the environmental complexity experiment, fish collected from the littoral site had significantly higher concentrations of maximum glucose and cortisol, and tended to have higher glucose and cortisol responsiveness, than limnetic fish. This indicates that although the collection site did not affect a fish's baseline values, intraspecific variation in site use is associated with divergent sensitivity of the hypothalamic–pituitary–interrenal axis to stressors. The importance of capture location on maximal response from stressors represents a potential sampling bias and source of variation, and may be even more pronounced in species that are habitat specialists.

**Key words:** pumpkinseed sunfish, *Lepomis gibbosus*, stress, social environment, habitat quality, cortisol, social buffering.

**Résumé :** Dans un contexte de changements planétaires, les réactions comportementales et physiologiques des animaux sauvages à des stressors suscitent un intérêt croissant. Si de nombreux facteurs peuvent influencer les réactions au stress, deux facteurs qui n'ont pas été étudiés en détail durant la phase induite par le stress sont la complexité environnementale et la présence d'individus conspécifiques. En utilisant des crapets-soleil (*Lepomis gibbosus* (L., 1758)) sauvages prélevés de sites limnétique et littoral, nous avons vérifié si la complexité environnementale et la densité des conspécifiques avaient une incidence sur le glucose et le cortisol durant la période de réaction maximum suivant un stressor normalisé d'exposition à l'air. En général, la complexité environnementale et la densité de conspécifiques n'avaient pas d'effet significatif sur le stress de maximal. Cependant, dans l'expérience sur la complexité environnementale, les poissons prélevés du site littoral présentaient des concentrations significativement plus élevées de glucose et de cortisol maximums et avaient tendance à présenter une plus grande réactivité du glucose et du cortisol que les poissons limnétiques. Cela indique que, bien que le lieu de prélèvement n'ait pas d'incidence sur les valeurs de référence d'un poisson donné, des variations intraspécifiques d'utilisation de sites sont associées à des sensibilités différentes de l'axe hypothalamique–pituitaire–interrénel aux stressors. L'importance du lieu de capture sur la réaction maximum à des stressors représente un biais d'échantillonnage et une source de variation possibles et pourrait même être plus prononcée chez les espèces spécialistes en matière d'habitat. [Traduit par la Rédaction]

**Mots-clés :** crapet-soleil, *Lepomis gibbosus*, stress, environnement social, qualité de l'habitat, effet tampon social.

## Introduction

The endocrine system of wild animals plays an important role in mediating life-history trade-offs and linking organisms to their environment (Zera and Harshman 2001; Ricklefs and Wikelski 2002). This is particularly true for glucocorticoid (GC) steroid hormones (Wingfield et al. 1998). GCs are present in all vertebrates and are the primary neuroendocrine stress response. GCs are released when the hypothalamic–pituitary–adrenal (HPA) axis (the hypothalamic–pituitary–interrenal (HPI) axis in fish) is activated in response to a perceived or actual stressor (Sapolsky et al. 2000). GCs also stimulate several secondary neuroendocrine responses including energy (glucose) mobilization (Wedemeyer et al. 1990). In the short term, the stress response is beneficial and necessary

to react appropriately to a challenge (Wingfield et al. 1998; Wingfield and Romero 2001), as it triggers physiological and behavioural changes that promote immediate survival and suspends activities that are temporarily unnecessary (Sapolsky et al. 2000). However, chronic activation of the HPA or HPI axis may be harmful to an individual's health, reproductive success, and survival (Romero et al. 2009; Boonstra 2013). Baseline-response values can be measured in the blood plasma if individuals are sampled rapidly (Gamperl et al. 1994; Romero and Reed 2005; Angelier et al. 2010), whereas maximum release in teleost fish typically occurs from 0.5 to 1 h later (Barton 2002). It is generally accepted that low baseline values of GCs (Busch and Hayward 2009; reviewed in Bonier et al. 2009) and a rapid increase in values followed by a

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**Table 1.** Stress-level proxies of pumpkinseed sunfish (*Lepomis gibbosus*) captured from littoral and limnetic sites in Lake Opinicon, Ontario, Canada, upon capture (baseline values) and during the stress-induced phase (maximum values) in four environmental treatments.

Variable	Environmental treatment			Capture site			Environmental treatment × capture site			Length		
	<i>F</i>	<i>df</i>	<i>P</i>	<i>F</i>	<i>df</i>	<i>P</i>	<i>F</i>	<i>df</i>	<i>P</i>	<i>F</i>	<i>df</i>	<i>P</i>
Baseline glucose concentration (mmol/L)	0.101	3, 108	0.960	1.550	1, 108	0.216	0.539	3, 108	0.656	2.492	1, 108	0.117
Maximum glucose concentration (mmol/L)	0.689	3, 108	0.561	4.668	1, 108	<b>0.033</b>	0.330	3, 108	0.803	0.081	1, 108	0.776
Glucose response concentration (mmol/L)	0.875	3, 108	0.457	3.832	1, 108	0.053	0.404	3, 108	0.750	0.016	1, 108	0.898
Baseline cortisol concentration (ng/mL)	1.497	3, 108	0.219	1.914	1, 108	0.169	1.162	3, 108	0.328	9.593	1, 108	<b>0.002</b>
Maximum cortisol concentration (ng/mL)	0.536	3, 108	0.659	4.451	1, 108	<b>0.037</b>	0.609	3, 108	0.610	6.810	1, 108	<b>0.010</b>
Cortisol response concentration (ng/mL)	0.406	3, 108	0.749	3.145	1, 108	0.079	1.369	3, 108	0.256	2.558	1, 108	0.113

Note: Responsiveness = maximum value – baseline value. Significant values ( $P < 0.05$ ) are set in boldface type, whereas near-significant values ( $0.05 < P < 0.1$ ) are set in italic type.

quick return to baseline levels (reviewed in Breuner et al. 2008) indicate higher fitness, though individual variation in coping styles interacting with environmental fluctuations may mediate these effects (Koolhaas et al. 2007; Cockrem 2013). This would enable an organism to respond appropriately to a challenge while limiting the negative consequences associated with the chronic elevation of GCs and secondary compounds (Angelier and Wingfield 2013).

Many factors affect how individuals respond to stressors and individual variation in response is affected by gene–environment interactions (Ellis et al. 2006). Individuals can vary in GC levels owing to intrinsic individual differences, such as reproductive status (Gesquiere et al. 2008), sex (Lance et al. 2001), and age (Walker et al. 2005). External factors can also affect the stress response and depend on the type, duration, and timing of their application. For example, red porgy (*Pagrus pagrus* (L., 1758)) adapted to black backgrounds had lower stress responses than those adapted to white backgrounds (Rotllant et al. 2003); red drum (*Sciaenops ocellatus* (L., 1766)) had higher stress responses to a longer restraint (Thomas and Robertson 1991); and green sturgeon (*Acipenser medirostris* Ayres, 1854) had higher stress responses at night than during the day (Lankford et al. 2003). Individuals can also vary in stress response owing to both long-term evolutionary processes (Hori et al. 2012; Pottinger and Carrick 1999), such as selective pressures from predators (Fischer et al. 2014), and short-term acclimation, such as 2 weeks in tanks of different colours (Gilham and Baker 1985). However, all those studies focused on environmental factors that were present before a stressor was applied, and thus, individuals had the opportunity to adjust to the new environment. It is possible that the environment experienced by an individual during the period of HPA or HPI activation following a stressor also influences the magnitude of the stress response, specifically the maximum concentrations. For example, wild rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792)) held for 1 h in a trap had higher maximum cortisol than those held in a stream enclosure (Clements et al. 2002); zebrafish (*Danio rerio* (Hamilton, 1822)) exposed to different coloured tanks for 0.5 h showed different maximum cortisol responses (Pavlidis et al. 2013); and brown trout (*Salmo trutta* L., 1758) transferred to novel tanks for 2 h had higher maximum cortisol than control fish (Lepage et al. 2000). Thus, the environments experienced by an individual prior to and after a stressor can affect the stress response.

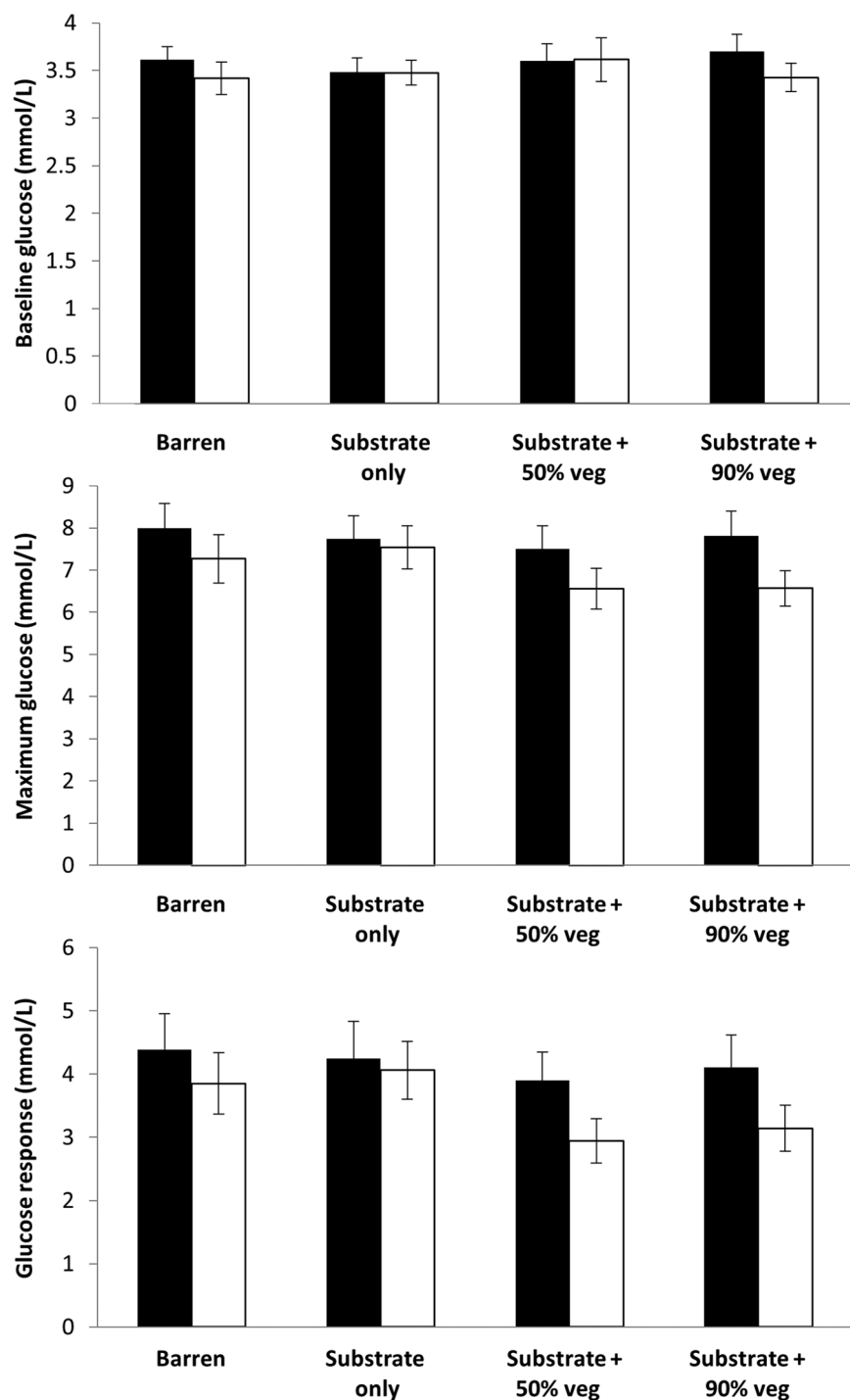
Anthropogenic effects, such as deforestation and fragmentation, often result in increased stress responses compared with less disturbed habitats (e.g., Wasser et al. 1997; Suorsa et al. 2003; Homan et al. 2003; reviewed in Jeffrey et al. 2015). Natural variation in habitat quality can also influence the HPA or HPI axis (Homyack 2010), as suggested by several observational studies (such as abiotic stability: Yamashita et al. 2003; temperature: Meka and McCormick 2005; food availability: Hellgren et al. 1993). Less is known about how habitat complexity, per se, influences stress (Naslund and Johnsson 2016). In jundiá (*Rhamdia quelen* (Quoy and Gaimard, 1824)), the presence of a shelter reduced the duration of the fish's stress response (Barcellos et al. 2009). In Atlantic salmon

(*Salmo salar* L., 1758), individuals raised in environments with complexity for 23 weeks had lower baseline cortisol concentrations compared with those in barren environments (Naslund et al. 2013). In contrast, zebrafish acclimated to enriched tanks for 7 days had higher baseline cortisol concentrations than those in barren environments (von Krogh et al. 2010); however, the initial housing complexity was not described. If the enriched tank was actually the novel environment (i.e., if zebrafish had previously been housed in barren tanks), the experimental duration may have been too short for fish to acclimate to the new environment. This is supported by a more recent study where zebrafish that had been raised in barren tanks for 5 months were transferred to barren or enriched tubs, and after 5 days, fish in barren tubs had lower baseline cortisol concentrations; however, at 10 days, the fish in enriched tanks had lower baseline cortisol (Keck et al. 2015). Thus, in general, more complex environments reduce the stress response, but this may depend on the duration in the environment and on the initial source of the individuals.

The social environment can also influence the stress response (Creel et al. 2013). Position in a social hierarchy can affect physiological responses; however, given the complexity of natural environments, these effects are often minor (reviewed in Sloman and Armstrong 2002). Isolation for a gregarious species, or interactions with conspecifics for a solitary species, can increase baseline GC levels (Hawkley et al. 2012). For example, high densities of conspecifics in zebrafish increase baseline cortisol responses (Ramsay et al. 2006); in Starlings (*Sturnus vulgaris* L., 1758), intruders that were part of a group of conspecifics had higher cortisol responses than solitary intruders (Nephew and Romero 2003); and baseline cortisol concentration in female pallid damselfish (*Pomacentrus amboinensis* Bleeker, 1868) was positively correlated with intruder density (McCormick 2006). Conversely, the presence of social companions can reduce the physiological reactions to a challenge, a phenomenon called social buffering (Hennessy et al. 2009; Hostinar et al. 2014). Although social buffering has been studied in mammals and birds, very few studies have focused on fish (but see Allen et al. 2009).

Pumpkinseed sunfish (*Lepomis gibbosus* (L., 1758); henceforth referred to as pumpkinseed) are typically found in the littoral zone feeding on hard-bodied prey (Osenberg et al. 1992), but in some lakes, they can also be found consuming zooplankton in offshore novel limnetic habitats (Robinson and Wilson 1996). Individuals found in the open habitat may have different physiological responses than those staying in the littoral habitat. In general, bold individuals have lower HPA or HPI reactivity than shy individuals (Schjolden et al. 2005; Cockrem 2007) because of shared genetic mechanisms affecting both physiology and behaviour (Overli et al. 2005). Specifically, bolder pumpkinseeds forage more in the pelagic water column and are less likely to swim close to conspecifics compared with shy pumpkinseeds (Wilson et al. 1993), and bolder congeneric bluegill sunfish (*Lepomis macrochirus* Rafinesque, 1819; henceforth referred to as bluegill) spend more time in the open water than shy bluegills (Wilson and Godin 2009; Wilson et al.

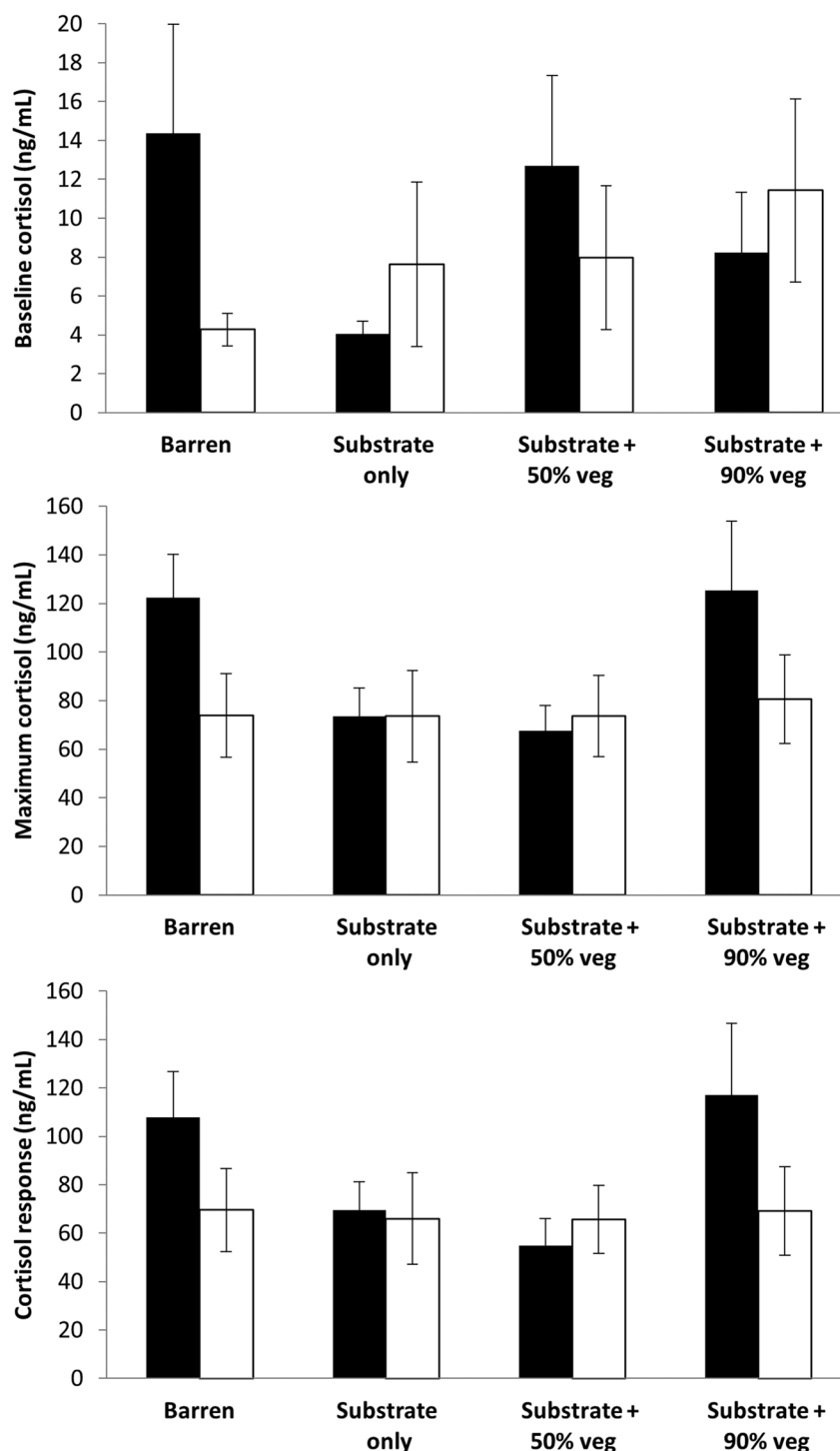
**Fig. 1.** Baseline and maximum glucose concentrations and glucose responsiveness for pumpkinseed sunfish (*Lepomis gibbosus*) collected from littoral (black bars) and limnetic (white bars) sites and assigned to four experimental environmental treatments (barren, cobble substrate, substrate with 50% vegetative (veg) cover, and substrate with 90% veg cover). Data presented are means  $\pm$  1 SE;  $n = 62$  and  $55$  for limnetic and littoral sites, respectively.



2011). Thus, pumpkinseeds found in littoral regions should have higher HPI reactivity than those in limnetic habitats. Additionally, littoral pumpkinseeds interact with the substrate and experience high levels of environmental structure (i.e., macrophytes) and large numbers of conspecifics in nearshore schools, whereas fish in limnetic regions are less likely to interact with the substrate in these open environments and are typically more solitary (Warren 2009). We captured sunfish in littoral and limnetic habitats to test whether capture environment influenced baseline or

maximal physiological response to a stressor. As the littoral habitat is the typical habitat for pumpkinseeds (Robinson et al. 1996), they are predicted to have lower baseline measures of stress than those in the novel and more exposed limnetic habitat. We then manipulated environmental complexity and conspecific density during the stress-induced recovery phase and predicted that the stress response would be magnified for fish that were “recovered” in environmental complexities and densities that were dissimilar to their capture location.

**Fig. 2.** Baseline and maximum cortisol concentrations and cortisol responsiveness for pumpkinseed sunfish (*Lepomis gibbosus*) collected from littoral (black bars) and limnetic (white bars) sites and assigned to four experimental environmental treatments (barren, cobble substrate, substrate with 50% vegetative (veg) cover, and substrate with 90% veg cover). Data presented are means  $\pm$  1 SE;  $n = 62$  and  $55$  for limnetic and littoral sites, respectively.



## Materials and methods

### Environmental complexity experiment

This study was performed from 26 June through 2 July 2014 at the Queen's University Biological Station located approximately 50 km north of Kingston, Ontario, Canada. Working from a 6 m research vessel, pumpkinseeds were collected by angling from Lake Opinicon, Ontario, Canada (44°56'N, 76°33'W), from both

limnetic and littoral locations. Locations were alternated to ensure fish were sampled in each location during both morning and afternoon to remove diurnal confounds on cortisol concentrations. Surface water temperature throughout the 9-day period remained fairly stable (25–28 °C) and mean temperatures were similar between habitats (littoral mean of 26.4 °C, limnetic mean of 25.8 °C). All fish were captured using small pieces of dew worm

**Table 2.** Stress-level proxies of pumpkinseed sunfish (*Lepomis gibbosus*) captured from littoral and limnetic sites in Lake Opinicon, Ontario, Canada, upon capture (baseline values) and during the stress-induced phase (maximum values) in three conspecific density treatments.

Variable	Density treatment			Capture site			Density treatment × capture site			Length		
	<i>F</i>	<i>df</i>	<i>P</i>	<i>F</i>	<i>df</i>	<i>P</i>	<i>F</i>	<i>df</i>	<i>P</i>	<i>F</i>	<i>df</i>	<i>P</i>
Baseline glucose concentration (mmol/L)	0.634	2, 94	0.533	0.752	1, 94	0.388	1.266	2, 94	0.287	2.969	1, 94	0.088
Maximum glucose concentration (mmol/L)	0.133	2, 94	0.876	0.083	1, 94	0.774	0.069	2, 94	0.934	0.002	1, 94	0.966
Glucose response concentration (mmol/L)	0.132	2, 94	0.876	0.046	1, 94	0.830	0.009	2, 94	0.991	0.238	1, 94	0.627
Baseline cortisol concentration (ng/mL)	2.606	2, 94	0.079	0.513	1, 94	0.476	2.521	2, 94	0.086	1.853	1, 94	0.177
Maximum cortisol concentration (ng/mL)	2.784	2, 94	0.067	0.335	1, 94	0.564	2.740	2, 94	0.070	14.810	1, 94	<b>0.000</b>
Cortisol response concentration (ng/mL)	2.364	2, 94	0.100	0.332	1, 94	0.566	1.767	2, 94	0.176	12.848	1, 94	<b>0.001</b>

Note: Responsiveness = maximum value – baseline value. Significant values ( $P < 0.05$ ) are set in boldface type, whereas near-significant values ( $0.05 < P < 0.1$ ) are set in italic type.

on barbless hooks. Only fish that were between 150 and 238 mm in length, hooked in the jaw, and showed no evidence of bleeding or other significant angling-related injury were included. All fish were fought for 10 s to standardize the capture stressor. Upon landing, the fish underwent a 3 min standardized air stressor. During the air-exposure period, a baseline blood sample was obtained using a 1 mL heparinized syringe and a 25 gauge, 1½ inch (38.1 mm) needle. Approximately 0.2–0.3 mL of blood was taken from the caudal vasculature, directly posterior to the anal fin. Any fish that was not bled within the 3 min air-exposure period was excluded from the experiment, because, in general, increases in cortisol concentrations are only detectable in teleost fish after that time (Pankhurst 2011); specifically, in pumpkinseeds, samples collected under 3 min represent baseline values (S.J. Cooke, unpublished data). Once the 3 min had elapsed, fish were introduced into one of four treatment tanks. All treatments took place in 68 L Rubbermaid bins (64.77 cm × 44.45 cm × 39.05 cm) filled to 30 cm with water collected from the lake that was replaced after every trial. The four treatments were (1) barren (a bin with just water), (2) a bin with cobble substrate (approximately 2.5 cm deep covering the bottom of the bin), (3) a bin with cobble substrate and approximately 50% vegetative cover collected from the lake (a combination of water milfoil (genus *Myriophyllum* L.) and pondweed (*Potamogeton* L.)), and (4) a bin with cobble substrate and approximately 90% vegetative cover. A lid with holes was placed on top of the bin and the assigned fish was left for 42 min (45 min total from start of air exposure). During that period, water temperatures remained stable and disturbance (e.g., boat motor noise, talking) was minimized. Working with the congeneric bluegill, Cook et al. (2012) reported that cortisol concentrations peaked at that time period. Following the 42 min, the fish were removed and a second blood sample was taken. If this second sample was not completed within 2 min, then the fish was removed from the study. This second blood sample represented maximum cortisol and glucose concentrations, whereas the first blood sample represented baseline concentrations.

Prior to being returned to the lake, total length (to the nearest millimetre) was recorded and the caudal fin was clipped in case of recapture. All blood samples were kept in the capped syringes and placed in an ice-water slurry for no longer than 2 h. Whole-blood glucose levels were read using a handheld glucose reader (Accu-Chek Compact Plus blood glucose meter; Roche Diagnostics) that had been previously validated for use on fish (Stoot et al. 2014). Blood samples were then centrifuged at 2000g for 5 min (Fisher Scientific Mini Centrifuge) and the resulting plasma was pipetted into two separate 1.5 mL screw-cap vials. Vials were kept in liquid nitrogen until they could be transferred to a –80 °C freezer where they remained until analysis.

### Conspecific density experiment

This study was also performed at the Queen's University Biological Station from 3 July through 6 July 2014. Pumpkinseeds were

collected using angling from Lake Opinicon from both limnetic and littoral locations as noted above. Surface water temperature throughout the 4-day period remained fairly constant (24–25 °C) and mean temperature was similar between habitats (littoral mean of 25.1 °C, limnetic mean of 24.5 °C). Blood sampling and sample processing was identical to the environmental complexity experiment, but here environment was standardized and only fish density was manipulated. Bins were left empty of substrate and vegetation (barren) and were filled to 30 cm with lake water. The three treatments were (1) no conspecifics, (2) one conspecific, and (3) five conspecifics that varied in length between 125 and 218 mm. Baseline and maximum glucose and cortisol samples were collected as above.

### Cortisol assays

Baseline and maximum plasma cortisol concentrations were assessed using a commercial radioimmunoassay kit (RIA; MP Biomedicals, Santa Ana, California, USA) and subsequently read with a 2480 WIZARD automatic gamma counter (PerkinElmer, Waltham, Massachusetts, USA) following the methods outlined by Gamperl et al. (1994). The intra- and inter-assay coefficients of variation were 8% and 10%, respectively.

### Statistical analysis

Stress responsiveness (for glucose and cortisol) was determined by subtracting the baseline value from the maximum value at 45 min (Cousineau et al. 2014). For the conspecific density experiment, we included responses from pumpkinseeds from the “barren” treatment of the environmental complexity experiment in the “no conspecific” treatment group because they experienced the same bin setup. A two-way ANCOVA was used to test treatment and capture-site effects for each experiment. We included length as a covariate because body size can affect cortisol concentrations (reviewed by Baker et al. 2013). Cortisol variables were transformed by  $\log(n + 1)$  to improve normality. Statistical analyses were performed using JMP version 5. Values are reported as means ± 1 SE.

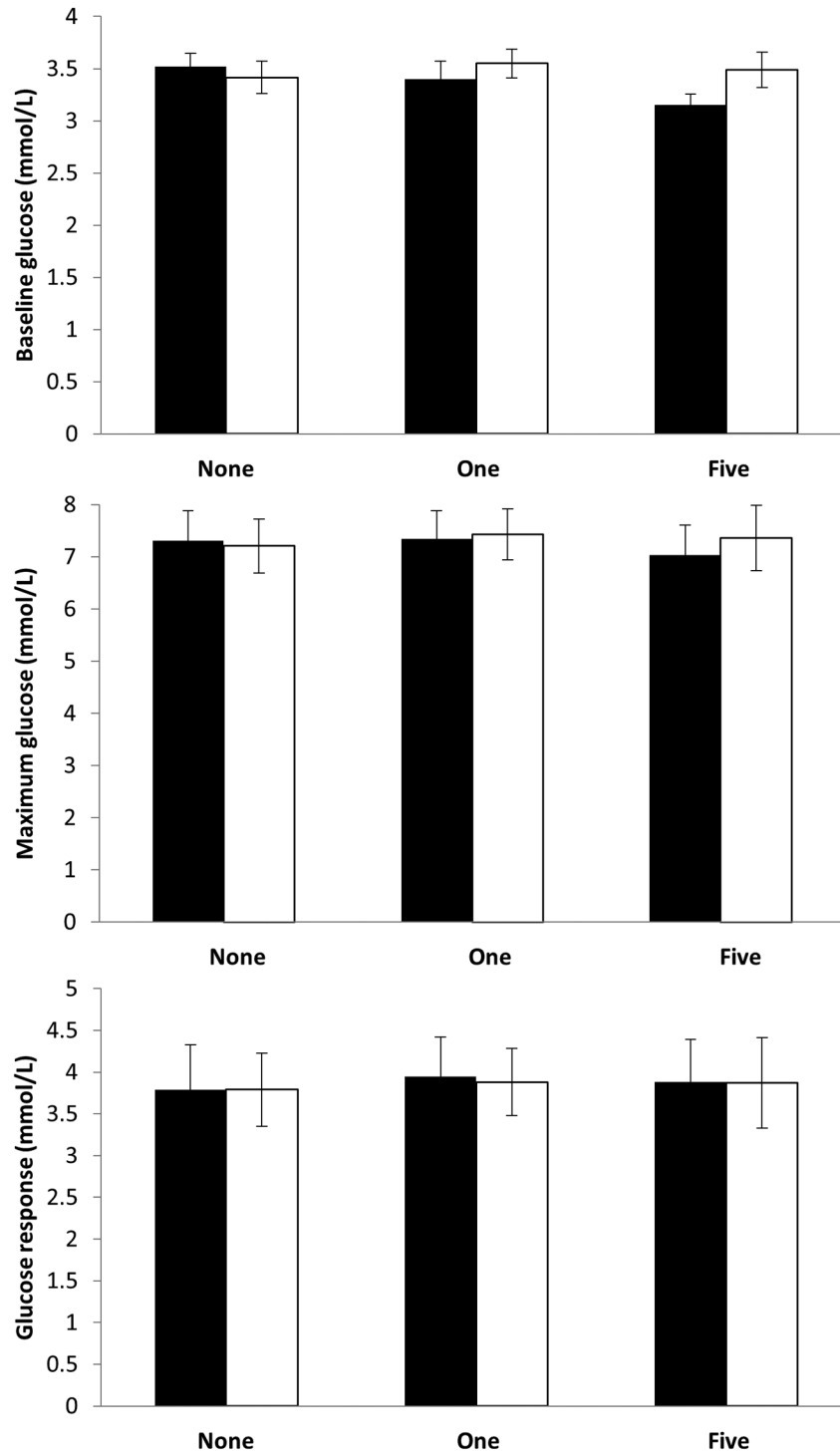
### Results

A total of 117 (62 limnetic and 55 littoral) and 101 (51 limnetic and 50 littoral) pumpkinseeds were used for the habitat and conspecific manipulations, respectively. Fish had similar total length across treatments for both environmental complexity ( $192 \pm 22$  mm) and conspecific density ( $190 \pm 20$  mm) experiments, as well as between source habitats (littoral:  $190 \pm 22$  mm; limnetic:  $192 \pm 21$  mm). In no case did the interaction between length and either capture site or treatment have a significant effect on cortisol concentrations (all  $P > 0.075$ ) and so these interactions were removed from the final analysis.

### Environmental complexity experiments

Pumpkinseeds collected from littoral habitats had significantly higher maximum glucose and cortisol concentrations, borderline significantly higher glucose responsiveness, and tended towards

**Fig. 3.** Baseline and maximum glucose concentrations and glucose responsiveness for pumpkinseed sunfish (*Lepomis gibbosus*) collected from littoral (black bars) and limnetic (white bars) sites and assigned to three conspecific density treatments (no other conspecifics (none), one other conspecific (one), and five other conspecifics (five)). Data presented are means  $\pm$  1 SE;  $n = 51$  and  $50$  for limnetic and littoral sites, respectively.



higher cortisol responsiveness compared with limnetic pumpkinseeds (Table 1; Figs. 1, 2). Treatment had no effect on any measure of glucose or cortisol concentration (all  $P > 0.22$ ; Table 1). Fish length was negatively related to baseline and maximum cortisol concentration (Table 1).

#### Conspecific density experiments

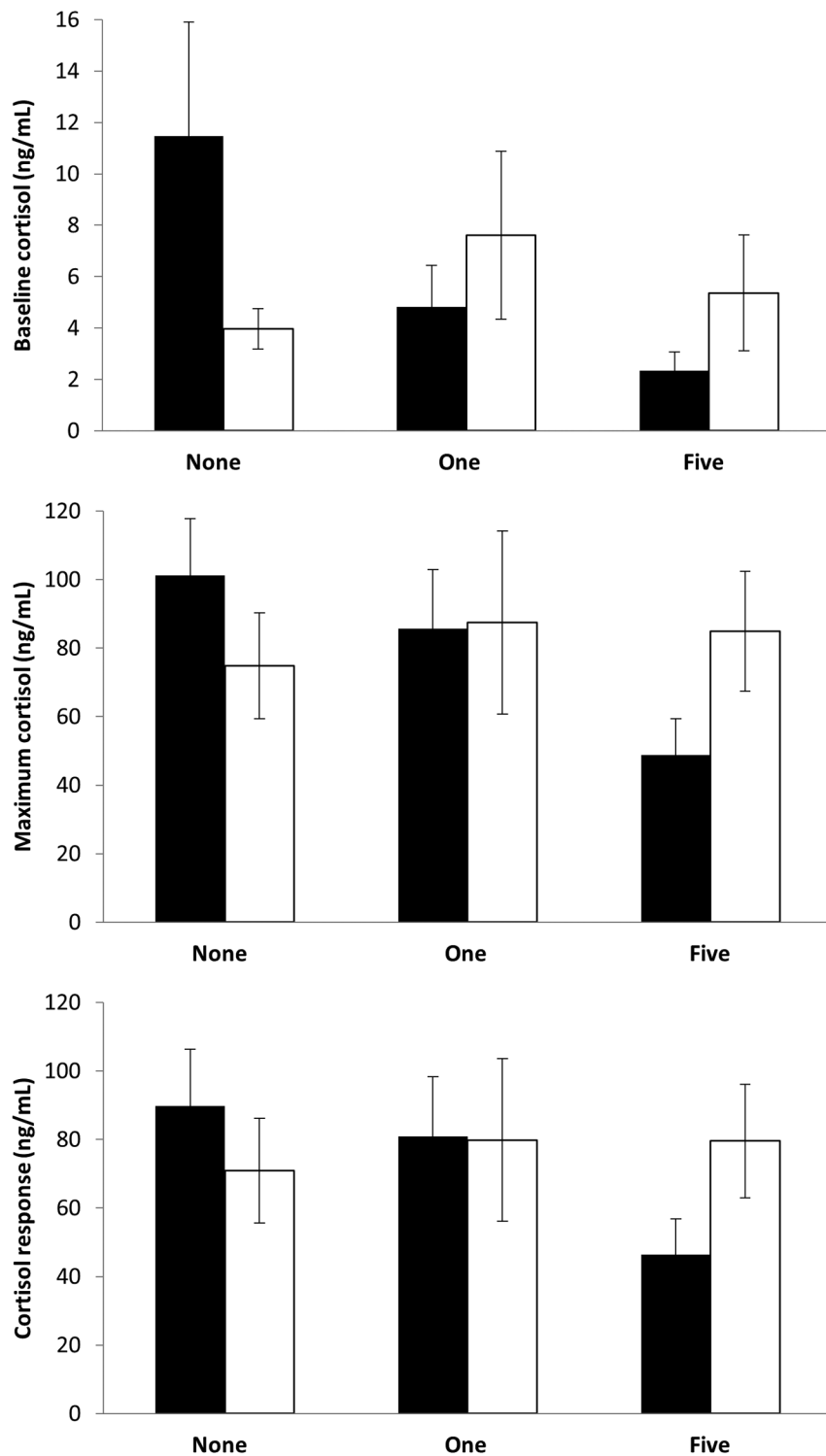
Littoral pumpkinseeds in the high density (five conspecifics) treatment tended to have the lowest maximum cortisol concen-

trations (capture site  $\times$  treatment,  $P = 0.0697$ ; Table 2; Fig. 4). No other factor affected any measure of either glucose or cortisol concentration (all  $P > 0.08$ ; Table 2; Fig. 3). Fish length was negatively related to maximum cortisol concentration and cortisol responsiveness (Table 2).

#### Discussion

The energetic requirements for basic maintenance may vary depending on habitat type. Open environments often have higher

**Fig. 4.** Baseline and maximum cortisol concentrations and cortisol responsiveness for pumpkinseed sunfish (*Lepomis gibbosus*) collected from littoral (black bars) and limnetic (white bars) sites and assigned to three conspecific density treatments (no other conspecifics (none), one other conspecific (one), and five other conspecifics (five)). Data presented are means  $\pm$  1 SE;  $n = 51$  and  $50$  for limnetic and littoral sites, respectively.



predation rates (Chipps et al. 2004), and in lakes with bluegills, pumpkinseeds also experience interspecific competition in the limnetic habitat (Mittelbach 1984), and so these fish were predicted to be more stressed than ones in littoral habitats. However, we found that baseline concentrations of glucose and cortisol did not differ between fish sourced from littoral and limnetic envi-

ronments, indicating that the two habitats impose the same level of baseline physiological stress in pumpkinseed. In contrast, higher maximum glucose and cortisol concentrations and the tendency for glucose and cortisol responsiveness to also be higher in littoral than limnetic fish indicates that the fish sourced from the two environments have different physiological responses to

challenges. Thus, even though Lake Opinicon contains only one littoral trophic morph of pumpkinseed (Berchtold et al. 2015), there is sufficient intraspecific variation within the littoral morph that leads to some individuals spending at least some time in the limnetic environment. It may be that individuals who venture into these open limnetic environments are bolder (Wilson and Godin 2009; Wilson et al. 2011) and that is linked with lower HPI reactivity. Similarly, bicolor damselfish (*Stegastes partitus* (Poey, 1868)) occupying exposed rubble habitat had lower maximum cortisol concentrations than those in the natural reef habitats (Schrandt and Lema 2011). However, we did not see the same effects of source habitat during the conspecific density experiment because here littoral fish exhibited lower maximum cortisol concentrations compared with the environmental complexity experiment. This may indicate that littoral fish are more sensitive to changes in habitat structure than to changes in density, which is consistent with a more flexible but generally higher HPI reactivity in littoral fish. Interestingly, the pattern of low maximum concentration values for littoral fish recovering from a stressor with a high density of conspecifics is consistent with the social buffering hypothesis, as littoral fish can interact with large numbers of conspecifics in nearshore schools (Warren 2009). However, we note this interaction did not reach statistical significance. Similarly, social lake sturgeons (*Acipenser fulvescens* Rafinesque, 1817) that were housed for 1 week in solitary tanks had higher cortisol responses and a longer stress response compared with individuals housed with conspecifics (Allen et al. 2009). The duration of the stress response was not assessed in our study, so whether this also occurs in pumpkinseeds is not known.

Responses to conspecific density may also depend on other factors. If there are different types of social hierarchies in littoral versus limnetic habitats, then the stress response may be affected by density in ways that we did not predict, but whether or not there are social hierarchy differences in the two habitats is not known. However, as we used similarly sized individuals and capture technique in both habitats, we assume that the average social position of individuals did not differ, and so would not affect our interpretation of habitat differences. Reproductive status may also affect responses to conspecifics, as male pumpkinseeds defend territories while females do not (Miller 1963). We did not determine sex of fish in our study and some males may still have been breeding (Colgan and Gross 1977; Brown 1985), so presumably we have a mixture of solitary males and more gregarious females. A stronger test of density effects would involve knowing the sex of the individual during breeding, or conducting the experiment when all individuals have ceased breeding. Additionally, we note that we only used one lake and so have no replication at the geographical scale (Hurlbert 1984).

An individual living in an optimal habitat is expected to better respond (i.e., a rapid increase in GCs with a quick return to a low baseline GC concentration) to a challenge than those living in degraded habitats (Wingfield 2005; Breuner et al. 2008; Bonier et al. 2009; Busch and Hayward 2009). Although most studies focus on factors that may affect an individual's response given enough acclimation time, we tested whether the environment experienced during the stress-induced phase itself would influence maximum concentrations of both primary and secondary neuroendocrine responses. This is the first test of the social buffering hypothesis during the stress-induced phase; although our results only hint at treatment effects, they warrant further investigation. In conjunction with the observed differences in HPI reactivity between littoral and limnetic pumpkinseeds during the environmental complexity experiment, we suggest that capture location is an important source of intraspecific variation and may represent a potential sampling bias if ignored, and that these patterns may be even more pronounced in species that are habitat specialists. With growing human disturbance affecting aquatic habitats around the globe (Dudgeon et al. 2006), understanding

the causes and consequences of intraspecific variation will allow us to link changes in habitat quality with microevolutionary processes and predict which individuals or species can cope with novel pressures (Angelier and Wingfield 2013).

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