Environ Biol Fish (2021) 104:989–1003
https://doi.org/10.1007/s10641-021-01133-4

Early life interactions with aquatic insects elicit physiological and behavioral stress responses in lake sturgeon (*Acipenser fulvescens*)

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Received: 18 March 2021 / Accepted: 1 August 2021

Abstract Predator encounters during early life can elicit behavioral and physiological responses that have fitness consequences during subsequent prey life stages. In threatened lake sturgeon (*Acipenser fulvescens*) and other lithophilic-spawning fishes, newly hatched larvae (free embryos) are exposed to abundant predators including aquatic insect larvae that co-occupy stream substrates. We investigated stress effects on lake sturgeon larvae after encounters with common aquatic insect predators by quantifying mortality, body size, cortisol levels, and swimming behavior. Free embryos were exposed to either Perlidae (stonefly obligate predators) or Isonychiidae (mayfly filterers and facultative predators) or to no predators (controls). Free embryos that encountered perlids experienced high mortality, elevated cortisol levels, and exhibited cortisol reactivity when subsequently exposed to an acute stressor. Free embryos that encountered isonychiids exhibited elevated mortality, and elevated cortisol and cortisol reactivity relative to controls. Findings indicate that lake sturgeon free embryos are stressed by exposure to members of benthic stream communities during early life stages (predation of nearby conspecifics), and that metrics of stress exhibited threat sensitivity. Data are consistent with predictions that individuals modulate antipredator behavior in response to the intensity of perceived predation threat in the environment. We determined that behavioral outcomes associated with encounters with aquatic insects altered future behavioral trajectories, potentially as an adaptive response that can affect predation rates in subsequent life stages. Results contribute to a broader understanding of how interspecies interactions among co-occurring predator and prey communities may impact individual fitness and fish population recruitment.

Keywords Aquatic invertebrates · Behavioral plasticity · Early life mortality · Lake sturgeon · Threat sensitivity

Introduction

Early life stress can have profound impacts on downstream behavior and physiology, with implications for future survival (Liesenjohann and Krause 2012; Middlemis et al. 2013; Chen et al. 2014). In particular, nonlethal predator effects can have notable influences on individuals, populations, and communities (Skelly and Werner 1990; Werner and Anholt 1996; Lima 1998; McCauley et al. 2011). Even in the absence of

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predator-induced mortality, the presence of predators can alter prey behavioral and physiological phenotypes (fish physiological state modulated by stress experienced in early life), and impact survival during subsequent life stages (Skelly and Werner 1990; Werner and Anholt 1996). For example, predator cues can alter the rate of early development (Mirza et al. 2001), or determine responses to subsequent predation cues (Ferrari and Chivers 2009). In fishes, exposure to conspecific alarm cues can be important by enabling individuals to respond to potential threats (Smith 1999; Vilhunen and Hirvonen 2003; Holmes and McCormick 2010). Therefore, understanding the role of early life predator exposure in individual development, and in mediating spatially and temporally varying prey perception and response (e.g., in the context of a landscape of fear; Gaynor et al. 2019), is important to predict population-level effects including prey dispersion and population stability (Sih 1987) and habitat selection (Gilliam and Fraser 1987).

Lake sturgeon (*Acipenser fulvescens*) are long-lived, iteroparous, lithophilic-spawning fishes that are native to northcentral North America (Peterson et al. 2007). Adults migrate from large lakes into rivers during the spring to spawn (Forsythe et al. 2012). Lake sturgeon adults spawn on hard gravel substrate, and free embryos burrow into interstitial spaces immediately after hatch until yolk sac reserves are depleted (Kempinger 1988) and larvae disperse (4–35 days post-hatch; Duong et al. 2011). During the free embryo and larval stages, lake sturgeon experience high levels of predation by invertebrates and fishes (Crossman et al. 2018; Waraniak et al. 2018). In response to conspecific predation, lake sturgeon are known to behaviorally react to the odor of injured or predated conspecifics during early ontogeny (Wishingrad et al. 2014a, b; Sloychuk et al. 2016). While it is unknown whether these odors constitute true alarm cues, as lake sturgeon larvae have not been shown to possess club cells (Shute et al. 2016), antipredator reactions to injured or predated conspecifics allow individuals to perceive and respond to predator presence (Laurila et al. 1997; Mourabit et al. 2010; Cao and Li 2020). During the lake sturgeon free embryo stage, olfactory perceptive abilities become functional prior to visual receptive ability (Dettlaff et al. 1993), enabling individuals to sense and responding to predator presence during early ontogenetic stages. However, non-lethal effects of predator presence on important stress-related physiology and behavior during early life stages after predator exposure for lake sturgeon or fish development generally have not been extensively investigated.

Early life stress, such as that induced by predator exposure (McCauley et al., 2011), can alter stress axis function, resulting in long-term changes in physiological and behavioral development (Schreck et al. 1997; Pariante and Lightman 2008; Piato et al. 2011; Jeanneteau et al. 2012). The hypothalamic-pituitary-inter-renal (HPI) stress axis becomes functional during the free embryo stage (the yolk sac larval stage after hatch) in many fish species including sturgeon (Simontacchi et al. 2009; Falahatkar et al. 2009). The stress axis is responsible for mediating physiological response to stressors via the release of cortisol, the stress hormone (Auperin and Geslin 2008; Lukkes et al. 2009). For lake sturgeon, early life predator-induced stress may have important downstream effects on future threat responses, especially during the larval drift period when lake sturgeon are particularly vulnerable to predation (Waraniak et al. 2018). Lake sturgeon larvae that experience early life stress in the form of chronically high temperature (Wassink et al. 2019) or higher egg cortisol levels (Wassink et al. 2020) have lower cortisol reactivity and higher activity levels that have been shown to be associated with higher survival in the presence of a crayfish predator, suggesting that early life stress has the potential to induce adaptive physiological and behavioral phenotypes.

Lake sturgeon free embryos and aquatic insect larvae are likely to interact due to co-occupancy of rocky substrate that are used for spawning, post-hatch rearing (lake sturgeon), and as preferred larval development habitats (certain aquatic insects) (Hamilton 2004). Larval stages of diverse aquatic benthic insect communities also occupy gravel substrate in streams (Bournaud et al. 1998; Jähnig and Lorenz 2008). Abundance and biodiversity of aquatic insects are heavily influenced by water quality and other environmental factors (Hershey et al. 2010; Dijkstra et al. 2014). Therefore, differing environments likely generate variation in taxonomic groups of aquatic insects encountered by lake sturgeon free embryos, as well as insect abundance and the frequency of such encounters. Members of the stonefly family Perlidae are predators of larval fish (Claire and Phillips 1968). Members of the mayfly family Isonychiidae are collector-filterers and facultative predators (Merritt et al. (2008). Therefore, depending on function, isonychiids could either potentially reduce stress of fish larvae by improving water quality (Menzie 1980; Morin et al. 1988) or increase stress and reduce survival via predation. Prior research with lake sturgeon eggs during incubation indicates that the presence of aquatic insects decreases the duration of incubation and concomitantly decreases body size at hatch depending on functional feeding group (Walquist et al., in review). Researchers lack understanding of how early life exposure to aquatic insects affects lake sturgeon after hatch during subsequent life stages.

This study investigated direct and indirect effects of lake sturgeon encounters with aquatic insect larvae during early ontogenetic stages. Specifically, we quantified mortality rates, body size, stress physiology, and swimming behavior of lake sturgeon larvae that encountered either predatory or non-predatory, or no insects during the free embryo stage. Threat sensitive responses are often tied to behavioral plasticity in the trade-offs between growth (foraging) and avoiding predation (Werner and Gilliam 1984). We hypothesized that exposure to perlids (predators) would cause alterations in behavioral and physiological reactivity since individuals were expected to encounter an environment of high predation-related stress. We predicted that lake sturgeon larvae exposed to perlids would have higher mortality rates, higher cortisol levels, and increased behavioral reactivity (such as increased activity levels) to alarm cue odors compared to larvae exposed to isonychiids or control larvae (no insect present). Understanding how interspecies interactions during early life stages impact lake sturgeon development will contribute to a broader understanding of proximal and long-term effects of biotic community interactions on wildlife species like lake sturgeon, a species of conservation concern, and whose populations are highly vulnerable to environmental changes.

Methods

Eggs and sperm were collected from adult sturgeon spawning in the Upper Black River in Onaway, MI on May 11, 2018. Eggs were fertilized using standard lake sturgeon hatchery procedures (Crossman et al. 2011; Bauman et al. 2016). All individuals used for the experiment were taken from one full sibling family, based on gamete fertilization using a single male and female to reduce variation due to family (genetic or maternal) effects (Dammerman et al. 2015; Wassink et al. 2020). Eggs were reared using a tumbling regime in McDonald egg-hatching jars (Pentair J32, Apopka, FL) until hatch. While tumbling jar rearing has been suggested to cause stress for developing embryos and increase cortisol levels (Earhart et al. 2020), all lake sturgeon in the experiment experienced the same rearing conditions during egg incubation, and thus tumbling jar rearing is unlikely to have affected outcomes among treatments. During the egg incubation stage, the mean (± SD) daily incubation temperature was 15.43 (± 0.53 °C). Free embryos were then moved to 3-l flow-through tanks supplied with stream water at ambient temperature (mean ± SD daily rearing temperature 17.0 ± 0.53 °C) and flow rate of 56.8 l/h. Stream water was sourced from the Upper Black River at ambient temperature throughout the experience, and therefore, lake sturgeon experienced the diel temperature variability and seasonal temperature increase that they would have experienced in the wild in their natal stream environment.

Experimental treatments using free embryo lake sturgeon began at 5 days after hatch, approximately 3 days prior to reaching the larval stage at the beginning of exogenous feeding (Fig. 1). Each of three treatments (perlid, isonychiid, and control) contained six replicate tanks (3-l aquaria), for a total of 18 tanks in the experiment, with each tank containing 25 sturgeon free embryos. In the control treatment, no insect was added to the 3-l tanks. In the isonychiid and perlid treatments, four aquatic insect larvae were added to each of the six replicate tanks in each treatment. All aquatic insect larvae were captured from the Upper Black River using either a kick-net or a D-frame drift net. Identification of insects to genera was conducted as described in Merritt et al. (2008) and included perlids as obligate predators (Plecoptera: Perlidae: genera Claassenia) and isonychiids as collector-filterer/facultative predator — Ephemeroptera: Isonychiidae: genera Isonychia. Free embryos were provided with Bioballs (2.54 cm^3 BioBalls Pentair #CBBI-5)

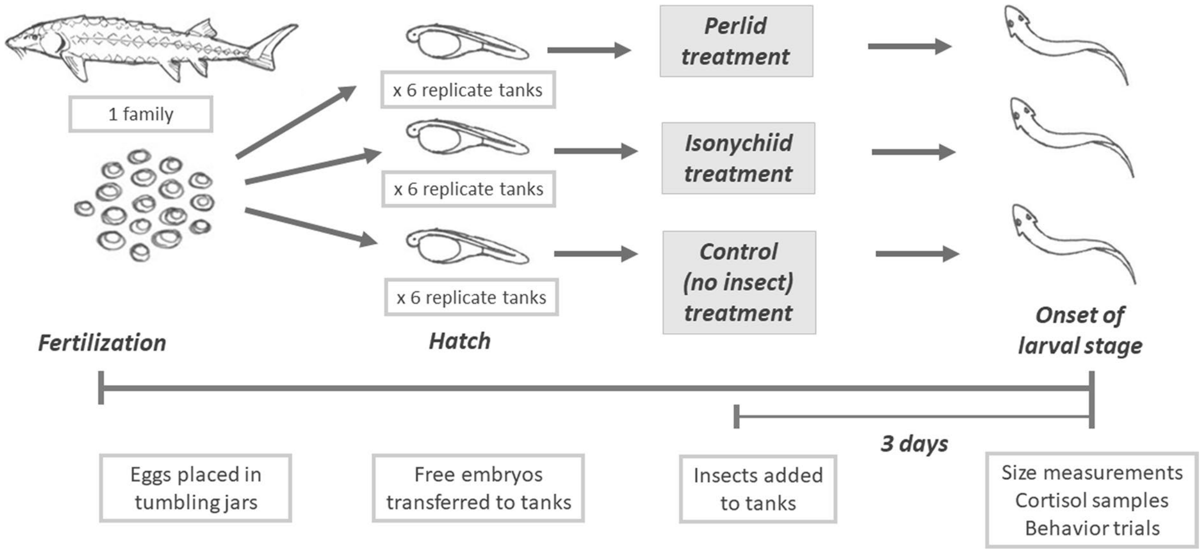

Fig. 1 Treatment diagram. All individuals used for the experiment were taken from one full sibling family. After fertilization, eggs were placed in McDonald egg-hatching jars (Pentair J32, Apopka, FL) until hatch. At hatch, lake sturgeon free embryos were transferred to 3-l flow-through tanks. Experimental treatments (perlid, isonychiid, or control) began at 5 days after hatch, approximately 3 days prior to the onset of the larval stage. Each of three treatments (perlid, isonychiid, and control) contained six replicate tanks (3-l aquaria), for a total of 18 tanks in the experiment, with each tank containing 25 sturgeon free embryos. In the isonychiid and perlid treatments, four aquatic insect larvae were added to each of the six replicate tanks in each treatment. In the control treatment, no insect was added to the 3-l tanks. Size measurements, cortisol samples, and behavior trials were conducted on the day the lake sturgeon were considered to have reached the larval stage (15 days post fertilization)

as artificial stream substrate. Food was not provided to lake sturgeon during the experiment, since lake sturgeon utilize yolk sac reserves during the free embryo stage and only begin exogenous feeding at the onset of the larval stage (approximately 8 days after hatch). For the isonychiid and perlid treatments, insects were left to interact with free embryos for 3 days and then removed from tanks. Lake sturgeon remained in the tanks until being removed for cortisol samples, behavior trials, and body size measurements that same day. All sampling for the experiment took place at the end of the 3-day period, which was the end of the free embryo stage and beginning of the larval stage (15 days post fertilization). The 3-day time period at the end of the free embryo stage was chosen since the HPI stress axis is functional by this time (Simontacchi et al. 2009), and because the subsequent onset of the larval stage is an important period in which antipredator behaviors influence survival rates (Wishingrad et al. 2014a, b; Crossman et al. 2018).

Free embryo mortality

After insects were removed from replicate tanks, all surviving lake sturgeon were counted to determine the mortality rate for each tank. Proportion of mortalities was calculated for each replicate. A beta regression and pairwise contrasts were used to determine if proportions of mortality differed among perlid, isonychiid, and control treatments using the *betareg* package (Cribari-Neto and Zeileis 2010) in program R v 3.2.2. (R Foundation for Statistical Computing, Vienna, Austria).

Larval body size

At the beginning of the lake sturgeon larval stage (15 days post fertilization), total body length (mm) of six individuals from each replicate in each of the three treatments was measured. Photos of lake sturgeon larvae were taken using a digital camera, and size measurements were made using ImageJ software

(National Institutes of Health, Bethesda, MD, USA, http://rsbweb.nih.gov/ij/). Images were used to quantify total body length for each individual using a ruler in each photo for calibration. A Shapiro–Wilk test indicated that the body size dataset was normally distributed ($p = 0.142$). A one-way ANOVA was conducted using the *aov* function in program R v 3.2.2. to determine differences in body size among treatments.

Larval cortisol levels

At the beginning of the lake sturgeon larval stage (15 days post fertilization), larval samples for cortisol analysis were taken at baseline (with no acute stressor applied) or after an acute stressor in order to quantify stress levels as well as physiological reactivity of the stress axis to an acute stressor. The acute stressor was a 20-s period of removal from the water and exposure to air which is a known stressor in sturgeon (Eslamloo and Falahatkar 2014). Afterwards, individuals were placed back in water for a rest period of 30 min before being euthanized. Each cortisol sample was a pooled sample of six individual larvae taken from one replicate tank. One baseline and one post-stress sample were taken from each replicate tank in the experiment, for a total of $n = 18$ baseline cortisol samples and $n = 18$ post-stress cortisol samples for the experiment. Individuals were euthanized using an overdose of MS-222 according to approved Michigan State University Animal Use and Care protocols. Whole-body cortisol levels were estimated using liquid chromatography tandem mass spectrometry with a Waters Xevo TQ-S mass spectrometer (Waters, Millford, MA, USA) as developed by our group for lake sturgeon (Bussy et al. 2017).

The cortisol data was not normally distributed, so log-transformation was applied prior to analysis. A Shapiro–Wilk test confirmed that the log-transformed dataset was normally distributed ($p = 0.818$). Generalized linear models were fit using the *glm* function in program R v 3.2.2. Predictor variables in the models included invertebrate treatment, stress state (baseline or after the acute stressor was applied), and the interaction of treatment and stress state. Body size was not included since each cortisol sample was a pooled sample of 6 individuals, whereas each size measurement was for a single individual. Variables present in the AICc selected model (Cavanaugh 1997) were further evaluated using an ANOVA and post hoc Tukey HSD tests.

Larval behavior

Behavior trials were conducted at the beginning of the larval stage (15 days post fertilization) for 6 individuals from each of the 6 replicate tanks in each insect treatment. Six larvae from each replicate tank were placed into a 15.24-cm-diameter petri dish filled with 50 micron filtered stream water. Larvae were allowed to acclimate to the petri dish for 1 min prior to beginning the video recording. Swimming activity was video recorded for 4 min using a Go-Pro Hero 4 camera (GoPro, Inc), without any additional stimuli added. After 4 min, video recording was paused and 1 ml of odor made from whole-body homogenization of conspecifics was added to the center of the petri dish using a pipette. Conspecific tissue homogenate has been shown to cause a physiological and behavioral response in sturgeon, which have an innate reaction to conspecific alarm cues released from skin (Wishingrad et al. 2014a, b). Homogenate was prepared by euthanizing 12 larvae using MS-222, rinsing thoroughly to remove residual MS-222, grinding whole-body tissue with a mortar and pestle, and filtering tissue through 100 ml of water to remove any tissue fragments. While injecting the odor into the petri dish, care was taken to position the pipette tip above the surface of the water, so that larvae would not react to the visual stimulus of the pipette top entering the water. Video recording was resumed for an additional 4 min to capture individuals' swimming activity in reaction to odor.

Loligo v.4.0 tracking software (Loligo Systems, Viborg, Denmark; https://www.loligosystems.com/software) was used to simultaneously track activity of the six individuals in each replicated trial, following Sakamoto et al. (2016). A center zone was defined that excluded a 1-inch perimeter around the petri dish edge to quantify edge-seeking behavior. Variables quantified from the entire 4-min video period included each individual's velocity (cm/s), acceleration (cm/s^2), percent time active, total distance traveled (cm), number of visits to center zone, and time (s) spent in center zone (Wassink et al. 2019, 2020).

Behavioral variables (percent activity, acceleration, velocity, distance, zone time, and number of center zone visits) were summarized into a composite behavioral measure using principal components analysis (Ballew et al. 2017). Datasets from videos prior to addition of alarm cue odor, with no stimulus, and datasets from videos after the addition of alarm cue odor were analyzed separately, since the same individuals were present in both sets of videos and thus data points were not independent. The broken stick method was used to determine that PC1, PC2, and PC3 were significant for the no stimulus dataset, and PC1 and PC2 were significant for the alarm cue dataset (Jackson 1993). Factor loadings above 0.5 were used to determine behavioral relevance of each principal component. ANOVAs were conducted using the *aov* function in program R v 3.2.2 for the principal components to determine whether there were significant differences among principal component scores from larvae from the perlid, isonychiid, and control treatments.

Results

Free embryo mortality

Mean percent mortalities among the six replicate tanks for the control treatment was 5.33%, for the isonychiid treatment was 7.33%, and for the perlid treatment was 18.67% (Fig. 2). The range of mortalities among replicates was 0–9 individuals. Beta regression and pairwise contrasts indicated that free embryo lake sturgeon from the perlid treatment experienced a significantly higher mortality rate than did the control treatment ($p = 0.003$). Mortality rates were intermediate for free embryos from the isonychiid treatment and relative to free embryos from either of the other treatments; however, differences were not significant ($p > 0.05$).

Larval body size

A Shapiro–Wilk test indicated that the body size dataset was normally distributed ($p = 0.142$). ANOVA indicated that treatment had a significant effect on body size at the beginning of the larval stage ($F_{2,105} = 6.37$, $p = 0.002$). Tukey HSD indicated that individuals in the perlid treatment were significantly larger (mean ± SD = 20.90 ± 1.63) than individuals from the other treatments (Fig. 3). No significant difference existed between lengths of individuals from the control treatment (mean ± SD = 19.76 ± 1.39) and the isonychiid treatment (mean ± SD = 19.87 ± 1.46) ($p > 0.05$; Fig. 3).

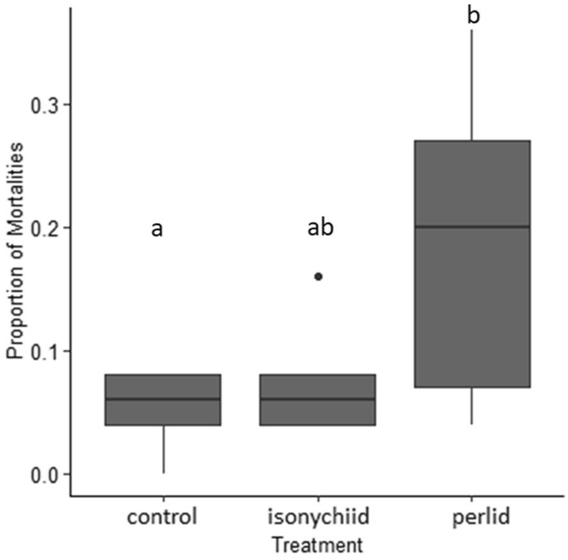

Fig. 2 Mean proportion of mortality associated with each aquatic insect treatment. Surviving larvae were counted after exposure to either no insects (control), isonychiid mayflies (isonychiid), or perlid stoneflies (perlid) for 3 days. Whiskers indicate minimum and maximum values, excluding data points beyond 1.5 × the interquartile range from upper and lower quartiles. Letters indicate significant difference among treatments based on post hoc pairwise contrasts

Larval cortisol levels

For both baseline and post-stress whole-body samples, larvae from the control treatment were characterized by the lowest cortisol levels (baseline mean cortisol 1.57 ± 0.50; post-stress mean cortisol 2 ± 0.35), larvae from the isonychiid treatment showed intermediate levels (baseline mean cortisol 1.83 ± 0.82; post-stress mean cortisol 3.67 ± 1.15), and larvae from the perlid treatment showed highest levels (baseline mean cortisol 2.72 ± 1.68; post-stress mean cortisol 5.97 ± 1.81). The AICc selected model included treatment, stress state, and the interaction of treatment and stress state (Table 1). ANOVA indicated a significant effect of treatment ($F_{2,33} = 22.08$, $p < 0.0001$), stress state ($F_{1,32} = 39.0$, $p < 0.0001$), and the interaction of treatment and stress state ($F_{2,30} = 3.34$, $p = 0.049$; Fig. 4). Tukey HSD indicated that individuals in the perlid and isonychiid treatments had significantly

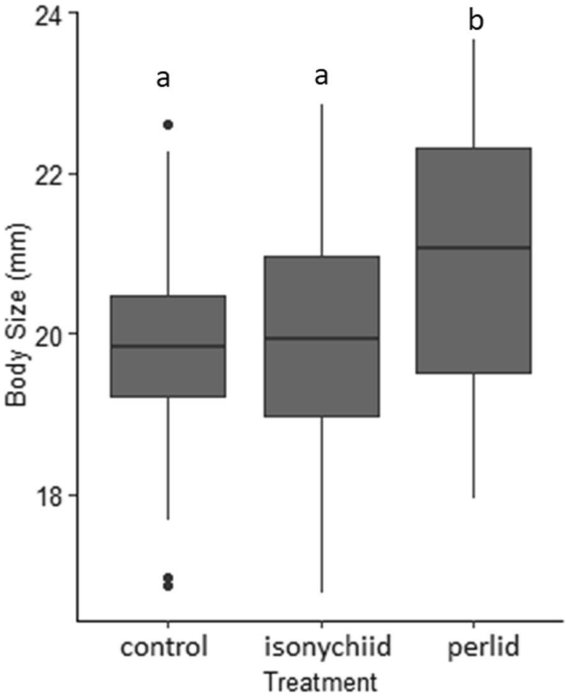

Fig. 3 Mean length (mm) of lake sturgeon at the beginning of the larval stage after exposure to aquatic insect treatment (no insects (control), isonychiid mayflies (isonychiid), or perlid stoneflies (perlid)). Sample sizes were 6 individuals per treatment, or 18 total larvae. Whiskers indicate minimum and maximum values, excluding data points beyond 1.5 × the range for upper and lower quartiles. Letters indicate significant difference among treatment means based on Tukey HSD post hoc tests

Table 1 Models for whole-body cortisol levels for lake sturgeon larvae exposed to isoncyhiids, perlids, or no insect at baseline and after an acute stressor

Model	AICc	Delta AICc	Weight
Cortisol ~ Treatment + Stress State + Treatment * Stress State	− 36.57	0	0.65
Cortisol ~ Treatment + Stress State	− 35.33	1.24	0.35
Cortisol ~ Stress State	− 12.14	24.44	0
Cortisol ~ Treatment	− 11.87	24.7	0
Null model	− 0.29	36.28	0

higher cortisol after an acute stressor. Tukey HSD also indicated that baseline cortisol levels for individuals exposed to perlids were significantly higher than those for larvae in the control treatment. Additionally, Tukey HSD indicated that post-stress cortisol levels for larvae exposed to perlids were significantly higher than cortisol levels from any other treatment or stress state (Fig. 4).

Larval behavior

Behavioral traits associated with swimming activity (percent activity, velocity, acceleration, distance, zone time, and zone visits) were reduced into three components using PCA for the no stimulus and alarm cue datasets. The broken stick method was used to determine which principal components were significant (Jackson 1993). PC1, PC2, and PC3 were significant for the no stimulus dataset, and PC1 and PC2 were significant for the alarm cue dataset.

For dataset of behavioral responses with no stimulus applied, factor loadings indicated that the most important variable contributing to variation along PC1 was percent activity. PC1, which explained 55.93% of the variation in the dataset, was positively associated with percent activity. ANOVA indicated a significant effect of treatment on PC1 ($F_{2,15} = 9.97$, $p = 0.0003$). A post hoc Tukey HSD test indicated that individuals from the perlid and control treatments were more active (higher mean PC1 scores) than individuals from the isonychiid treatment. PC2 explained 25.48% of the variation in the dataset and was negatively associated with velocity, acceleration, distance, and zone visits. ANOVA indicated a significant effect of treatment on PC2 ($F_{2,15} = 6.81$, $p = 0.0017$). A post hoc Tukey HSD test indicated that individuals from the perlid and control treatments moved slower, traveled smaller distances, and avoided the center zone more (higher mean PC2 scores) compared to individuals from the isonychiid treatment (Fig. 5, Table 2).

For the dataset of behavioral responses after individuals were exposed to alarm cue odor, PC1 explained 49.16% of the variation in the dataset, and factor loadings indicated PC1 was negatively associated with distance and zone visits. ANOVA indicated a significant effect of treatment on PC1 ($F_{2,15} = 4.45$, $p = 0.0142$). A post hoc Tukey HSD test conducted for PC1 indicated that individuals from the isonychiid treatment traveled smaller distances and avoided the center zone more (higher mean PC1 score) compared to individuals from perlid treatment, in the presence of alarm cues. PC2 explained 25.53% of the variation in the dataset and was negatively associated

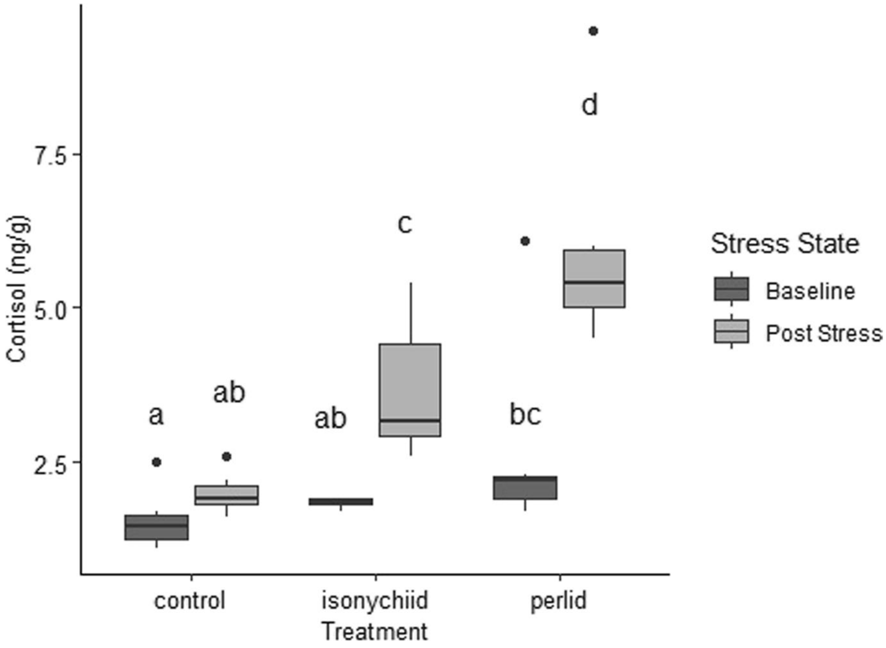

Fig. 4 Cortisol levels presented in original scale, for lake sturgeon at the beginning of the larval stage after exposure to aquatic insect treatments (no insects (control), isonychiid mayflies (isonychiid), or perlid stoneflies (perlid)). Cortisol levels are shown at baseline (pre exposure to air exposure as an acute stressor) and following exposure to the acute stressor. Letters indicate significant difference among treatment means based on Tukey HSD post hoc tests. Whiskers indicate minimum and maximum values, excluding data points beyond 1.5 × the range for upper and lower quartiles

with velocity and acceleration. ANOVA indicated a significant effect of treatment on PC2 ($F_{2,15} = 5.99$, $p = 0.0035$). A post hoc Tukey HSD test conducted for PC2 indicated that individuals from the perlid treatment moved slower (higher mean PC2 score) than individuals from the isonychiid treatment, in the presence of alarm cues. PC3 explained 17.76% of the variation in the dataset and was positively associated with zone time and negatively associated with percent activity. ANOVA conducted for PC3 indicated no significant differences among treatments ($F_{2,15} = 2.05$, $p = 0.134$) (Fig. 5, Table 2).

Discussion

This study demonstrated that aquatic insect presence had important effects on lake sturgeon physiology and behavior during early life stages after hatch. Specifically, results showed that lake sturgeon free embryos experienced higher mortality in the presence of perlids relative to the no insect treatment, with predation as the most likely cause of death based on observation. Thus, mortality results confirm the importance of perlids as a lake sturgeon predator. Additionally, the non-zero mortality rate of lake sturgeon free embryos in the presence of isonychiids implicates isonychiids as facultative sturgeon predators. Based on whole-body cortisol levels, lake sturgeon demonstrated threat sensitivity, whereby alterations to physiological phenotypes differed depending on the aquatic insect group encountered. Perlid encounters induced relatively high stress, with elevated baseline and post-stress cortisol levels, while isonychiid encounters elevate stress levels slightly compared to controls (Fig. 4). Interactions with aquatic insects also had downstream effects as seen by variation in lake sturgeon larval swimming behavior among insect treatment groups, both with and without the presence of alarm cues (Fig. 5). Findings collectively highlight the importance of community-level factors, including predator encounters, on phenotypes and survival.

Predator-induced stress reactivity has been documented elsewhere. For example, in *Brachyrhaphis episcopi*, a freshwater fish, individuals from areas of higher predation risk had reduced physiological reactivity to stress, likely as an adaptation allowing individuals to function despite high stress environments (Archard et al. 2012). Similarly, tadpoles (*Rana sylvatica* and *Rana clamitans*) exposed to alarm cues showed lower physiological reactivity (suppression of the stress axis), likely as a means of promoting behavioral quiescence in order to avoid detection by predators (Fraker et al. 2009). In contrast, this study showed that lake sturgeon exposed to predation risk exhibit increased physiological

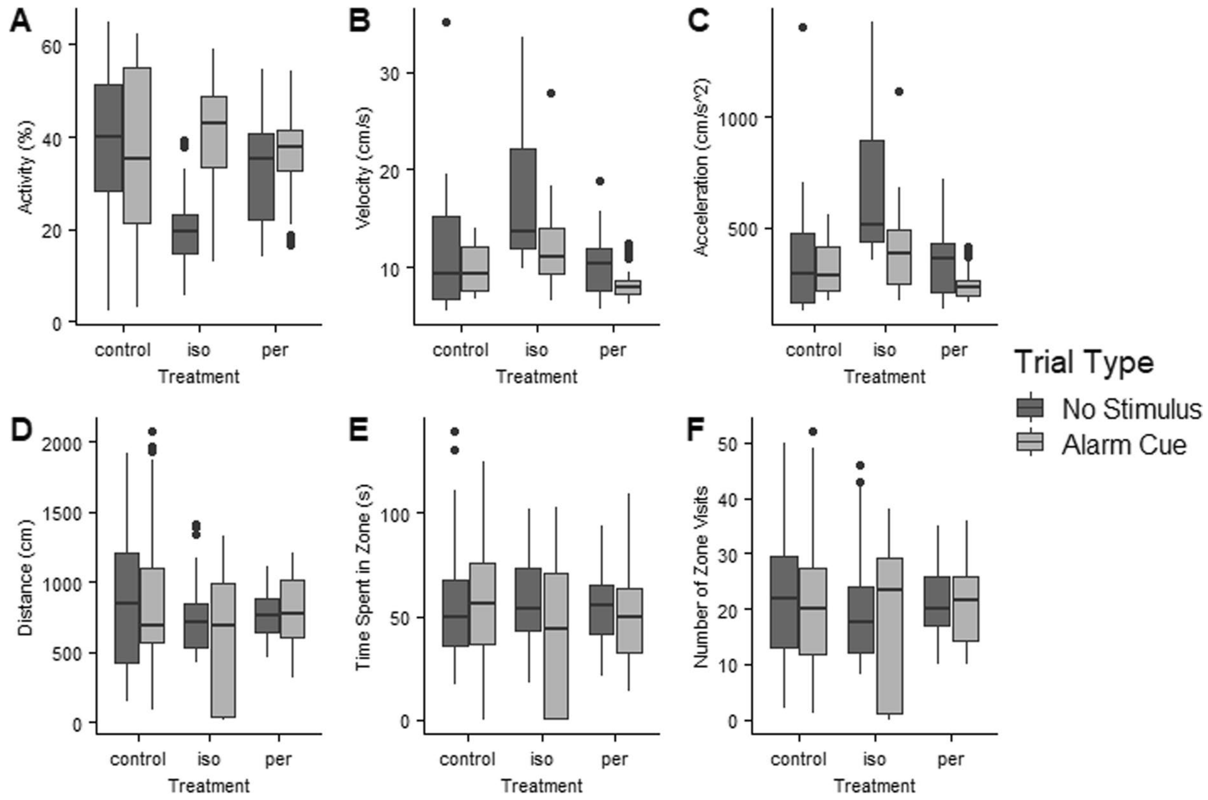

Fig. 5 Behavioral responses of lake sturgeon at the beginning of the larval stage after exposure to aquatic insect treatments (no insects (control), isonychiid mayflies (iso), or perlid stoneflies (per)), in the absence of stimuli, and after the addition of an alarm cue odor. Loligo software was used to extract % time active (**A**), velocity (cm/s) (**B**), acceleration (cm/s^2) (**C**), total distance traveled (cm) (**D**), time spent in center zone (s) (**E**), and number of visits to center zone (**F**) for each individual in each video. Whiskers indicate minimum and maximum values, excluding data points beyond 1.5 × the range for upper and lower quartiles

reactivity to an acute stressor. It is possible that stress-induced changes in physiological reactivity depend on the specific stressor experienced. This study used air exposure as a standardized acute stressor to observe stress axis function, and did not directly observe cortisol elevation in response to alarm cues. Therefore, it is possible that the intensified stress axis reactivity exhibited by lake sturgeon experiencing high predation risk applies to some acute stressors (such as air exposure) but not others (such as alarm cues). Another alternative explanation is that alarm cues are not acute stressors, but rather information, whereas direct interaction with predators is acute stress. Research into differential responses of the stress axis to different threats likely to be encountered in nature could provide insight into physiological stress reactivity as a means of adaptation in response to environments experienced by individuals. It has been suggested that elevated cortisol does not necessarily directly align with increased stress levels in wildlife, as cortisol comprises only part of the complex physiology of stress responses (Romero and Beattie 2021). Further research is necessary to determine role of cortisol for lake sturgeon in mediating ecologically important functions such as antipredator behaviors. Higher cortisol levels should not be assumed to be inherently positive or negative, as cortisol is important in mediating adaptive physiological and behavioral phenotypes (Wassink et al. 2019; Romero and Beattie 2021). Still, results indicate that interspecies interactions do have the potential to influence physiological stress responses via changes in cortisol levels.

Table 2 Factor loadings and eigenvalues for principal components analysis of swimming behavior with no stimulus applied and after the addition of alarm cue odor

	Factor loadings						Eigenvalue	Variance %	Cumulative variance %
	Velocity	*Accel*	*Zone time*	*% activity*	*Distance*	*Zone visits*			
No stimulus applied									
PC1	−0.4168	−0.4338	0.2308	0.5095	0.4003	0.4062	3.3560	55.9332	55.9332
PC2	−0.5139	−0.4841	−0.0993	−0.0028	−0.5001	−0.4915	1.5289	25.4812	81.4145
PC3	0.0267	0.0294	0.9599	−0.1936	−0.1914	−0.0550	0.8726	14.5436	95.9581
PC4	−0.2556	−0.2030	−0.1174	−0.7258	−0.0904	0.5871	0.1540	2.5666	98.5247
PC5	−0.1172	−0.1405	0.0417	−0.4188	0.7375	−0.4955	0.0858	1.4308	99.9555
PC6	−0.6945	0.7181	0.0081	0.0267	0.0313	−0.0147	0.0027	0.0445	100.0000
Alarm cues added									
PC1	0.3901	0.4380	−0.3477	−0.3197	−0.4599	−0.4705	2.9495	49.1578	49.1578
PC2	−0.5943	−0.5240	−0.0570	−0.2115	−0.4215	−0.3828	1.5319	25.5317	74.6895
PC3	0.0594	0.0816	0.6960	−0.6952	−0.0556	0.1378	1.0654	17.7564	92.4458
PC4	−0.1060	−0.1004	−0.6182	−0.5987	0.3438	0.3462	0.3173	5.2880	97.7338
PC5	0.0300	−0.0496	0.0956	−0.1024	0.6956	−0.7023	0.1305	2.1745	99.9083
PC6	0.6921	−0.7172	−0.0116	−0.0290	−0.0754	0.0081	0.0055	0.0917	100.0000

Interpretation of body length differences among lake sturgeon larvae from different treatments is limited, since length measurements prior to exposure to aquatic insects are not available. Without a before and after comparison, the significantly larger size of individuals from the perlid treatment cannot be conclusively linked to interaction with perlids despite the fact that all individuals were full siblings. However, prior studies have shown that in larval lake sturgeon, larger size aids predator avoidance (Crossman et al. 2018; Wassink et al. 2019), supporting the interpretation that smaller individuals experienced higher rates of predation by perlids. Lake sturgeon body size during early ontogeny is closely tied to water temperature, with individuals reared in warm temperatures having much faster development but with smaller sizes (Kempinger 1988; Smith and King 2005). Temperature could therefore influence lake sturgeon susceptibility to predation by aquatic insects. Specifically, warmer temperatures could increase rates of predation by decreasing mean size of lake sturgeon free embryos. Further investigation could investigate the inter-relationship of temperature, size, and fitness in the context of lake sturgeon and invertebrate communities (Kingsolver and Huey 2008).

Interaction with aquatic insects during the free embryo stage also influenced swimming behavior of larval lake sturgeon (Fig. 5). Comparison of individuals' behavior before and after addition of alarm cue odor to the petri dish is limited, since different behavioral variables informed variation along the principal component axes of each analysis. However, results indicated that swimming behavior differed among lake sturgeon larvae from different treatments, both with and without the presence of alarm cue odor. Based on PC1 from analysis of behavior with no stimulus applied, individuals from the isonychiid treatment had lower activity levels, while individuals from perlid and control treatments had similar swimming activity. Prior studies have indicated that high activity levels are associated with higher survival in the presence of crayfish predators (Wassink et al. 2019); however, in this study, the higher activity levels are unlikely to be an adaptive response to predator experience since they were observed in individuals from the control treatment. Notably, behavior results did not match cortisol results, with intensity of behavioral differences proportional to the amount of predation experienced. One possible explanation is that the rate at which lake sturgeon encountered potential predators, resulting in direct interaction, was more important the amount of nearby conspecifics predation (indirect interaction) in determining behaviors. Individuals that encountered perlids frequently were more likely to be predated and thus removed from the sample population prior to behavior trials. Further investigation could examine whether interaction with less-predatory species has a larger impact on a population's mean behavioral phenotypes due to higher survival rates for individuals that experience such interactions.

Based on PC1 from analysis of behavior in the presence of alarm cue odor, individuals from the isonychiid treatment traveled shorter distances and avoided the center zone more, suggesting they were more likely to avoid alarm cues which were added to the center of the petri dish. This result indicates that even when predator-induced mortality was low, as was the case with the isonychiid treatment, interaction with aquatic insects appears to affect larval lake sturgeons' reaction to alarm cues. Avoidance of alarm cues is likely an adaptive behavior in the presence of predators (Laurila et al. 1997; Mourabit et al. 2010; Cao and Li 2020). It should be noted that no control substance was included in the experimental design, so it is also possible that lake sturgeon were reacting to the stimulus of an odor being introduced into the environment, rather than reacting specifically to alarm cue odor as a signal of imminent predation threat. Regardless, lake sturgeon from the isonychiid treatment have altered behavioral responses to stimuli after interacting with isonychiids during the free embryo stage. Interspecies interaction may therefore induce adaptive behavioral development that allows individuals to avoid predation despite low prior experience with predation risk during periods of high vulnerability to predation.

Individuals that did experience high levels of nearby conspecific predation moved slower (based on PC2 from analyses of behavior with no stimulus and with alarm cues) regardless of whether alarm cue odor was present (Fig. 5). Potentially, slower speeds could be an adaptive response to threat exhibited both in the novel environment of the petri dish, which has been shown to be a stressor for lake sturgeon larvae (Wassink et al. 2019, 2020), as well as in response to alarm cues. Another possibility is that lake sturgeon larvae that had been actively avoiding perlids had reduced energy, resulting in slower movements during behavior trials. Juvenile lake sturgeon with chronic predator exposure have been shown to have depressed flight responses to threats and spend more time occupying safer environments that offer more opportunities to hide from predators (Bjornson et al. 2020). The decreased swimming speeds and disregard for alarm cue odor we observed may therefore be related to individuals' risk assessment, informed by increased perceived threat levels associated with chronic predator exposure (in the perlid treatment). Bjornson et al. (2020) suggest that lake sturgeon behavioral responses take into account multiple risks simultaneously to produce optimal fitness outcomes, especially in relation to foraging. While we did not investigate foraging behavior in our study, lake sturgeon could have been responding to chronic predator risk by limiting energy expenditure in a new high-risk environment (the novel environment of the petri dish) as an adaptive response to promote maximum survival in high-risk conditions. Conclusions about adaptive or maladaptive outcomes of interactions with aquatic insects require further investigation that quantifies survival rates during subsequent life stages.

Interestingly, while behavioral responses in this experiment did not intensify with level of predation threat, cortisol level did reflect threat level experienced in a pattern predicted by the threat sensitivity hypothesis (Helfman 1989). The threat sensitivity hypothesis states that individuals will adjust their behavior in response to the intensity of the perceived predation risk in the environment (Helfman 1989). First, the three insect experimental exposures represent a gradient in predation threat intensity as expected based on knowledge of insect functional feeding guilds [Merritt et al. 2008 (none, low (isonychiids), and high (perlids)]. Data indicated that cortisol, as a measure of stress level, tracked the threat gradient. While the threat sensitivity hypothesis primarily makes predictions about behavioral responses to threat, our results suggest that physiology may also align with predictions. In this experiment, cortisol did not directly measure what the animals experienced such as numbers of predator encounters, chases, attacks, and duration of exposure to alarm cues, as defined in more traditional behavioral studies. However, cortisol levels and intensity of cortisol responses to an acute stressor varied in accordance with the level of predator-induced mortality being experienced by nearby conspecifics during an earlier life stage (Fig. 4), as predicted by the threat sensitivity hypothesis. Therefore, whole-body cortisol levels appear to represent an accurate relative measure of predator threat experienced, and could be considered a measure of "fear" level of individuals based on threat-related stimuli.

To gain further understanding of how aquatic insect larvae influence lake sturgeon survival, studies on how environmental factors influence aquatic insect predatory behavior would be valuable. Perlids hunt as a searcher in dark conditions, but an ambusher in light conditions (Sjöström 1985). Since free embryos are burrowed into substrate, they may be more likely to fall prey to perlids hunting as searchers in dark conditions, so abiotic factors such as moon phase and cloud cover could also influence rates of perlid predation on lake sturgeon. Further research could investigate under what conditions isonychiids switch from filter-feeding to predation, in order to predict effects on lake sturgeon free embryo survival. For example, temperature influences adult size of isonychiids (Wallace and Merritt 1980), which could determine the feasibility of preying on lake sturgeon free embryos. Environmental variables are likely important for determining not only the abundance and biodiversity of aquatic insects encountered by lake sturgeon free embryos but also whether such encounters result in predation.

In conclusion, findings of this study broadly indicate that interspecies interactions have important effects on sturgeon development, and thus a community-level perspective is important for understanding sources and outcomes of early life stress. Further research could profitably explore how stress-altered physiological and behavioral phenotypes associated with exposure to aquatic insect larvae during the free embryo stage influence later predation rates of sturgeon, for example, when larvae have dispersed from substrates inhabited by the obligate and facultative predators investigated here. Generally, one important question deserving further attention is whether organisms that experience early life stress, exhibit stress reactivity, and stress sensitivity (Helfman 1989) can navigate a landscape of fear (Gaynor et al. 2019) during subsequent life stages when exposed to novel threats and in contextually different environments. Moving forward, understanding implications of stress-related phenotypes for wildlife populations require a thorough understanding of interspecies interactions within ecological systems.

Acknowledgements We thank the 2018 Black Lake Sturgeon Facility crew for technical assistance: K. Marciano, S. Valentine, E. Rutledge, K. Skubik, and J. Kimmel. K. Holekamp, M. Wagner, and members of the Scribner laboratory provided useful comments on earlier drafts of the manuscript.

Author contribution L.W., D.L., W.L., and K.S. conceived the ideas and designed methodology; L.W., J.R., B.H., G.J., and D.L. collected the data; L.W. and D.L. analyzed the data; and L.W. and K.S. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Funding Support for this project was provided by the Michigan Department of Natural Resources (MDNR), the Great Lakes Fishery Trust, the U.S. Fish and Wildlife Service Coastal Program, and Michigan State University Ag Bio Research.

Availability of data and material Data and R-code from this project has been deposited and is available for review at our lab's GitHub site at https://github.com/ScribnerLab/Insect_Cortisol/. A doi number will be established upon acceptance of the manuscript.

Code availability ImageJ is freely available on the NIH website (https://imagej.nih.gov/ij/). R is freely available for download on the R Project website (https://www.r-project.org/).

Declarations

Ethics approval All experiments were conducted under approved Michigan State University Animal Use and Care protocols (04/17–071-00). To the extent possible, discomfort was minimized for all individuals in the experiment. Adults captured for gamete collection were handled for about 4 min each, while head and gills remained underwater. In the hatchery, Bioballs were provided as artificial substrate for free embryos until emergence. Once reaching the larval stage and beginning exogenous feeding, individuals were supplied with food ad libitum (premium grade brine shrimp Artemia sp., BSEP16Z, Brine Shrimp Direct). Flow rate of filtered stream water in tanks was maintained at 56.78 l/h to ensure adequate oxygenation. During the 4-min behavior trials in petri dishes, larvae were supplied with oxygenated stream water for the duration of the trial. Any larvae sacrificed for cortisol samples or for alarm cue odors were euthanized according to approved Michigan State University Animal Use and Care protocols using an overdose of MS-222.

Consent to participate Not applicable as this research did not involve human participants.

Consent for publication The authors all agree with the content of the manuscript, give explicit consent to submit for publication, and have permission from responsible authorities at Michigan State University (where the work was carried out) to submit this manuscript for publication.

Conflict of interest The authors declare no competing interests.

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