



## INVITED PAPER

# Coping With Urban Habitats Via Glucocorticoid Regulation: Physiology, Behavior, and Life History in Stream Fishes

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**Synopsis** As environments become urbanized, tolerant species become more prevalent. The physiological, behavioral and life-history mechanisms associated with the success of such species in urbanized habitats are not well understood, especially in freshwater ecosystems. Here, we examined the glucocorticoid (GC) profiles, life-history traits, and behavior of two species of fish across a gradient of urbanization to understand coping capacity and associated trade-offs. We studied the tolerant live-bearing Western Mosquitofish (*Gambusia affinis*) for two years and the slightly less tolerant, egg-laying, Blacktail Shiner (*Cyprinella venusta*) for one year. We used a water-borne hormone method to examine baseline, stress-induced, and recovery cortisol release rates across six streams with differing degrees of urbanization. We also measured life-history traits related to reproduction, and for *G. affinis*, we measured shoaling behavior and individual activity in a novel arena. Both species showed a trend for reduced stress responsiveness in more urbanized streams, accompanied by higher reproductive output. Although not all populations fit this trend, these results suggest that GC suppression may be adaptive for coping with urban habitats. In *G. affinis*, GC recovery increased with urbanization, and individuals with the lowest stress response and highest recovery had the greatest reproductive allotment, suggesting that rapid return to baseline GC levels is also an important coping mechanism. In *G. affinis*, urban populations showed altered life-history trade-offs whereas behavioral traits did not vary systematically with urbanization. Thus, these tolerant species of fish may cope with anthropogenically modified streams by altering their GC profiles and life-history trade-offs. These results contribute to understanding the mechanisms driving species-specific adaptations and thereby community structure in freshwater systems associated with land-use converted areas.

## Introduction

Anthropogenic alterations to habitat through land-use conversion contribute significantly to wildlife population extinctions and loss of biodiversity (Turner et al. 2007; Brooke Mde et al. 2008; Ceballos et al. 2015). Changes to natural habitats associated with urbanization are generally drastic and rapid (i.e., human-induced rapid environmental change [HIREC], sensu [Sih et al. 2011]) and can result in the persistence of only tolerant species in urban habitats. Many studies have examined the responses of terrestrial species to urbanization (Sol et al. 2013; Abolins-Abols et al. 2016;

Bonier and Martin 2016; Ibanez-Alamo et al. 2020), but fewer studies have explored the mechanisms of how HIREC affects populations of aquatic species (Jeffrey et al. 2015; King et al. 2016; Santana Marques et al. 2020). The freshwater biome, which includes over 40% of Earth's fish biodiversity, is especially sensitive to landscape modifications (Ricciardi and Rasmussen 1999; Lundberg et al. 2000; Gabor et al. 2018). Freshwater fishes are among the taxa most imperiled by the effects of land-use conversion, and worldwide 25% of freshwater fishes are at risk of extinction (Miller et al. 1989; Ricciardi and Rasmussen 1999; Vié et al. 2009). The

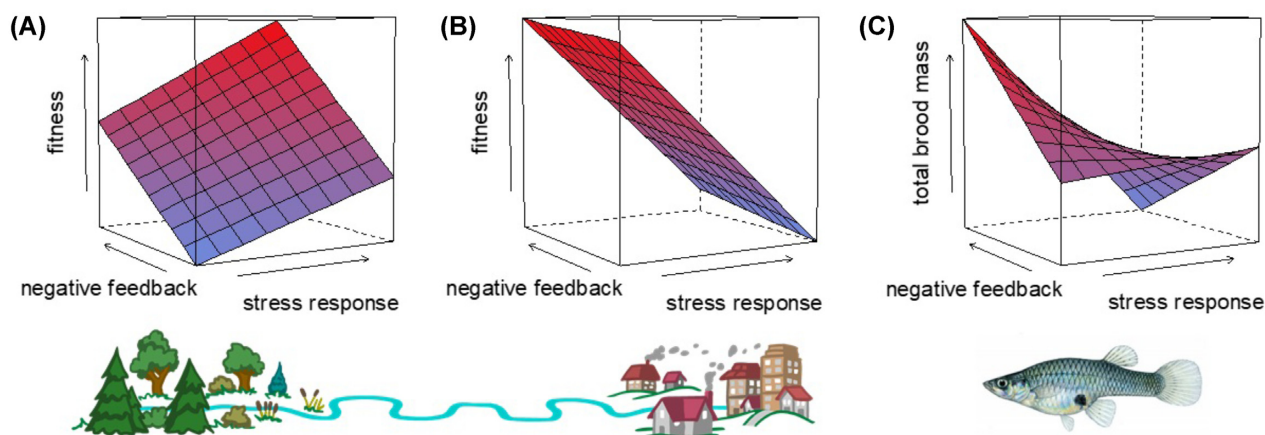
ecological changes brought about by streams draining urban catchments are collectively known as “the urban stream syndrome,” including altered hydrology, elevated temperatures and concentrations of nutrients and contaminants, reduced biotic richness, and the presence or dominance of more tolerant species (Karr 1986; Paul and Meyer 2001; Rahel 2002; Meyer et al. 2005; Walsh et al. 2005).

There is a wide range of phenotypic differences between organisms in urban populations and their conspecific counterparts living in non-urban habitats, including differences in morphology, physiology, behavior, and life history (reviewed by Fraker et al. 2002; Bonier 2012; Sol et al. 2013; Seress and Liker 2015; French et al. 2018; Gabor et al. 2018; Sepp et al. 2018). Most of this knowledge comes from research on terrestrial taxa, although some efforts have been made toward understanding how urbanization affects aquatic organisms (Brans et al. 2018a; Brans et al. 2018b; Brans et al. 2018c; Limburg and Schmidt 1990; Kern and Langerhans 2018; Côte et al. 2021). To date, little is known about the mechanisms by which tolerant species cope with degraded streams, and we are still far from fully understanding how urban environmental changes result in divergent phenotypes with respect to non-urban streams and rivers (Marques et al. 2019). Key attributes associated with fish species successfully surviving or thriving in degraded habitats include physiological tolerances and life-history traits that enhance survival and reproduction in potentially stressful urban habitats (Ricciardi and Rasmussen 1998).

Endocrine systems facilitate the ability of organisms to respond to and interact with their environment and play a role in species adapting to urban habitats (Partecke et al. 2006; Bonier 2012; Dantzer et al. 2014; Jeffrey et al. 2015; Ouyang et al. 2019; Ibanez-Alamo et al. 2020). In particular, glucocorticoid (GC) hormones produced by the hypothalamic-pituitary-interrenal (HPI) axis mediate the response of vertebrates to both predictable and unpredictable changes in the environment (Romero et al. 2009; Guindre-Parker 2018), thereby facilitating physiological, behavioral, and morphological responses to environmental perturbations (Wingfield and Kitaysky 2002). In response to acute stressors, cortisol (the primary GC in fish) is transiently elevated, helping maintain homeostasis by temporarily increasing energy metabolism, maximizing oxygen uptake during low oxygen conditions (McDonald et al. 1991), and moderating immune and reproductive functionality (Wendelaar Bonga 1997; Barton 2002; Romero 2004). The dynamic GC response to acute stressors is ultimately self-regulated through negative feedback, allowing organisms to return to baseline GC levels and maintain normal physiological

processes (Sapolsky 1983; Dallman et al. 1992). When perturbations persist over long periods of time, elevated GCs can have pathological effects including altered behavior, and negative fitness consequences, which can lead to death (Wingfield and Sapolsky 2003). The relationships between stress response, negative feedback, fitness, and how these relationships change depending on the degree of environmental perturbation are not yet understood (but see Vitousek et al. 2019). In general, effectively coping with stressors should involve a balance between mounting a robust GC response and effectively terminating the response (negative feedback) to return to normal behaviors and physiological processes (Wingfield 2013; Vitousek et al. 2019). Therefore, the highest fitness may be associated with a robust stress response and fast negative feedback (Fig. 1A), as has been found in birds (Vitousek et al. 2019). In urban habitats, however, animals are exposed to many stressors including disturbance by humans, noise pollution, artificial light at night, and toxic chemicals, and therefore they may dampen their stress responsiveness as this may minimize the fitness-reducing effects of prolonged or frequent stressors (Partecke et al. 2006; Bonier 2012). In this case, the highest fitness may be achieved by individuals with the lowest stress response (Fig. 1B). It is currently unknown whether the physiology of urban fishes relies on any of these two mechanisms to cope with anthropogenic environments.

Urbanization may also influence life-history traits, *via* changes in various ecological factors including food availability, population density, predation intensity, temperature, and concentrations of toxic compounds (Brans et al. 2018a; Johnson and Bagley 2011; Santana Marques et al. 2020). For example, high availability of nutrients in eutrophic urban streams may allow females to increase fecundity even above that expected for their body size because abundant nutrient-rich food would support simultaneously body growth, self-maintenance, and offspring production (Kuzuhara et al. 2019). In addition, if predation risk is low in urban streams, due to reduced abundance and diversity of predators, then carrying numerous eggs or embryos does not entail a high risk of mortality for reproductive females, such as would be expected in undisturbed environments with relatively high predation rates (Ghalambor et al. 2004). Thus, some freshwater species that thrive in urban settings may exhibit a disproportionately high reproductive investment, indicating that urbanization could promote a steeper relationship between body size and fecundity. Similarly, there is often a trade-off between the size and number of offspring (Stearns 1989; Roff 2002; Frias-Alvarez et al. 2014), and this trade-off may be alleviated in food-rich anthropogenic



**Fig. 1** Predicted (A, B) and observed (C) relationships of fitness with stress response and negative feedback. In natural habitats (A), combination of strong stress response and strong negative feedback is expected to show the highest fitness. In urban habitats (B), suppressed stress response may be favored. In *Gambusia affinis* we found the highest reproductive allotment in individuals with low stress response and strong negative feedback regardless of the intensity of urbanization (C). Sketch of the urban gradient by Zoltán Simanovszky; picture of *G. affinis* from Fishes of Texas Project.

environments (Snell-Rood et al. 2015; Santana Marques et al. 2020).

Behavioral changes are also often observed in the altered environments of urban habitats, mostly in the form of more risk-prone behaviors (Sih et al. 2011; Miranda et al. 2013; French et al. 2018). Behavioral traits like high activity and exploration may be favored during various stages of urbanization (Sih et al. 2012; Sol et al. 2013; Polverino et al. 2018). For example, colonization of urban habitats is facilitated by dispersal, which in turn is facilitated by behavioral types that are more active, more explorative and take more risks (Cote et al. 2010; Sol et al. 2013). These behavioral traits may also facilitate population growth in colonized habitats as individuals with these traits also tend to be more successful in competing for resources and therefore, grow faster and reproduce earlier (Cote et al. 2010; Polverino et al. 2018). In fish, sociability (shoaling behavior) may also influence how they react to human presence (Samia et al. 2019). Overall, however, little is known about the effects of urban stream syndrome on behavioral traits (Wenger et al. 2009).

In this study, we assessed the effects of urbanization on the physiology, life history and behavior of the Western Mosquitofish, *Gambusia affinis*, a globally invasive and tolerant species of live-bearing freshwater fish (Linam et al. 2002; Pyke 2005; Whittier et al. 2007). First, we examined GC profiles across a gradient of urbanization, including baseline cortisol release rates, stress response, and recovery from a stressor as a measure of negative feedback. Second, we investigated the following life-history traits and how urbanization modifies their patterns of covariation: reproductive allotment (total brood mass), fecundity (number of offspring), mass of individual offspring, and female

body size. Third, we analyzed the relationship between GCs and reproductive allotment as a proxy for fitness, and we explored whether this relationship varied with urbanization to test if individuals in different habitats cope with stressors by different mechanisms (Fig. 1A and B). Fourth, we tested whether the populations differed in behavioral traits related to risk taking, exploration, activity, and sociability (shoaling). Additionally, we studied the GC physiology and life-history traits of another less widespread but tolerant freshwater species of egg-laying minnow, the Blacktail Shiner, *Cyprinella venusta*, a fish with persistent or increasing abundances in systems altered by dams and agriculture land use practices (Walser and Bart Jr 1999; Meador and Carlisle 2007). Our non-manipulative approach of examining GC physiology, life-history traits, and behavior across the gradient of urbanization may help elucidate how tolerant species succeed and sometimes become invasive in disturbed freshwater habitats.

## Materials and methods

### Study species

*Gambusia affinis* are small live-bearing fish in the family Poeciliidae, native to much of the eastern USA. They are now invasive and present worldwide. Females typically mature in 1–2 months and can live up to 1.5 years (Pyke 2005). Young are typically born after 21–28 days of gestation (Krumholz 1948). Depending on body size, a female can produce roughly 14–218 embryos per brood and can produce up to 6 broods throughout the reproductive season of March–October (Krumholz 1948; Haynes and Cashner 1995). There are significant differences in the size and number of offspring of female



*G. affinis* across habitats (Stearns 1983; Reznick et al. 1990).

*Cyprinella venusta* are small egg-laying fish in the family Cyprinidae. They are found in the southeastern USA (Page and Burr 1991). They live up to 4.5 years (Littrell 2006). In Texas, spawning typically occurs from April to September (Littrell 2006). Females are sexually mature within the first year, produce egg clutches of 139–459 eggs (Page and Burr 1991), and are capable of spawning 24–46 clutches throughout the reproductive season (Baker et al. 1994). The timing of reproduction of female *C. venusta* can be affected by habitat disturbance and, in addition, the size of their ova decreases in disturbed environments, suggesting that their life-history traits vary depending on the degree of habitat perturbation (Casten and Johnston 2008).

### Field collection

All procedures in this study were in accordance with animal ethics guidelines and approved by the Texas State University IACUC (#83). Fish were collected under a Texas Parks and Wildlife Scientific Research Permit. We collected fish from six streams located within the Edward's Plateau region of Central Texas (Fig. S1; Table S1). We collected *G. affinis* and *C. venusta* from four streams from 22 May to 12 June 2018. In 2019, we only collected *G. affinis* from four streams (to focus on the GC profile and due to difficulties with *C. venusta*). Due to heavy rainfall (average of 49.9 cm in 2019 compared to 27.7 cm from March–June in 2018; US Climate Data; Austin, TX), we could not sample until 22 June to 2 July 2019. The two most rural streams used in 2018 no longer had an abundance of *G. affinis* in 2019, therefore two new sites were selected in 2019 along with the two other sites previously sampled in 2018. We determined the degree of urbanization by the % of developed land in the subwatershed surrounding each stream sampling site (Table S1), as quantified by the % of impervious surface cover (Paul and Meyer 2001; Walsh et al. 2005), using the USGS's 2011 national land cover dataset (NLCD 2011) in ArcMap 10.6.1 (ESRI). Impervious surface cover is an accurate predictor of urbanization and urban impacts on streams (McMahon and Cuffney 2000), and many report that the onset of ecological degradation is associated with 10–20% impervious surface cover of the catchment area (Paul and Meyer 2001).

At each site, we collected female *G. affinis* (sample sizes per site, 2018: N = 20; 2019: N = 18) and *C. venusta* (2018; N = 16) using dip nets and seines for water-borne hormone sampling in the field (see section below). We then collected additional (see sample sizes below) female *G. affinis* (both years) and *C. venusta* (2018 only) and placed them in breathable

bags for transportation to the laboratory for behavior and life-history studies. At each site, we also obtained a point measure of water temperature, pH, salinity, conductivity, total dissolved solids, and nitrates (2019 only), using hand-held water quality meters (YSI Inc.; Table S1).

### Measuring GC profiles

We collected individual cortisol release rates via a non-invasive water-borne hormone sampling technique (Following: Scott and Ellis 2007; Blake and Gabor 2014; Blake et al. 2015) in the field. Within 20 min of capturing with dip net, we placed each individual female *G. affinis* into sterile 250 ml beakers containing 100 ml of spring water. For *C. venusta*, we placed each individual into a 400 ml sterile beaker with 200 ml of spring water. Each beaker contained a low-density polyethylene (LDPE) plastic liner with opaque wall and lid and with holes on the bottom to easily transfer fish between beakers for repeated measures. Each fish remained in their beaker for 30 min to obtain baseline cortisol release rates. Following 30 min, we transferred the liner with the fish to a second sterile 250 ml beaker containing 100 ml of spring water. After moving the fish to the second beaker we agitated each fish by gently shaking it for 1 min every other min for a total duration of 30 min to obtain cortisol release rates in response to acute stress (agitation). We also measured post-agitation cortisol recovery rates of *G. affinis* (2019 only) by moving the fish to a third sterile 250 ml beaker with 100 ml of spring water and allowing the fish to remain in the beaker for 1 h. We transferred water samples to individual high-density polyethylene (HDPE) sample cups and stored them on ice. We then euthanized each fish by placing them in an ice-water slurry and measured the standard length (SL) of each fish to the nearest 0.1 mm using dial calipers and stored the fish in 70% ethanol for subsequent life-history analysis. Once in the laboratory, we stored water-borne hormone samples at −20 °C for future processing.

### Life-history traits

In both years, we dissected each female *G. affinis* (2018: N = 50–53/site; 2019: N = 44–47/site) used previously for measuring GC profiles or behavior, removed their broods, recorded gestational stage, and then calculated fecundity as the total number of eggs (stages 1–3) and embryos (stage 4+) per fish (following Haynes 1995). For *C. venusta* (N = 15–43/site) we removed their ovaries. Counting of eggs was not feasible in this species because the eggs in the ovaries were no longer clearly visible due to dehydration, as our storage method prioritized DNA integrity over structural integrity of

the eggs. We then dried the broods (eggs and embryos in *G. affinis*, ovaries in *C. venusta*) and the eviscerated fish for 48 h at 55 °C. We weighed the dried broods and eviscerated specimens (mg) using an analytical scale. We calculated total reproductive allotment (RA) as the total dry mass of all combined eggs and embryos per female *G. affinis* or total ovary dry mass for *C. venusta*. For *G. affinis* we calculated individual offspring dry mass by dividing the total dry mass of all combined eggs and embryos by the total number of eggs and embryos per female fish.

### Behavior of *Gambusia affinis*

In 2018 and 2019, we housed up to 30 female *G. affinis* per site (2018: N = 30–39/site; 2019: N = 15–21/site) in 37.85 L aquaria after collection. We kept fish on a 14L:10D cycle at 25°C and fed them tropical fish flakes (TetraMin) once daily. Following 40–50 h, we transferred 5 fish into a new container for behavioral observations. In 2018, this was a 37.85 L tank (51 × 25 × 31 cm) covered on all sides with dark-tinted glass, for 10 min acclimation. The tank was filled with dechlorinated water, approximately 5 cm from the bottom to minimize vertical column movement. After the 10 min of acclimation, we remotely filmed fish shoaling from above for a total of 10 min with a 1.3 MP webcam (Dynex Inc.). In 2019, for the behavioral observations individual fish were transferred to an opaque container (9 × 9 × 18 cm) filled with dechlorinated water and containing a square cutout for a door (5 × 5 cm) that was hinged to the lid connected to monofilament line. The container served as a refuge and was placed in the corner of a shallow opaque plastic white tub (52 × 35 cm) containing 8 cm of dechlorinated water to restrict vertical movement. We mounted a webcam above each tub to record trials. Fish acclimated in the refuge for 5 min, and then we remotely opened the door by pulling on the fishing line from the other side of the room. We ended the trial 5 min after the fish left the refuge, or if the fish did not leave the refuge after 5 min of observation. After recording individual behavior, we recorded shoaling behavior by transferring 4 fish into an opaque (29 × 16 cm) container filled with 6 cm of treated water and immediately recorded behavior with a webcam mounted above. We recorded shoaling behavior for 5 min. We decreased the number of fish per group compared to the 2018 experiment to optimize sample size and to match prior publications on shoaling (Tobler and Schlupp 2006) and decreased trial time based on our results from 2018.

After the behavioral recordings in each year, we euthanized individual fish in an ice-water slurry, and stored each individual in 70% ethanol for life-history

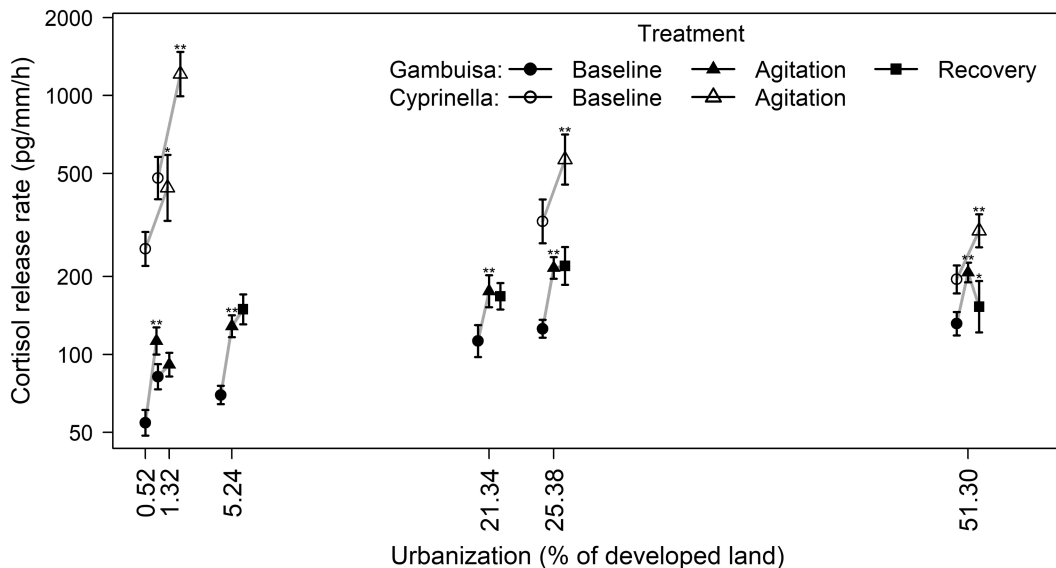
analysis. We used video-tracking software (Ethovision XT version 14; Noldus Information Technologies Inc.) to quantify individual behavior which included time spent moving (s), distance moved (cm), and velocity (cm/s). In 2019, we also measured the latency (s) to emerge and stay out for at least 10 consecutive seconds in the novel environment. We also quantified shoaling behavior by measuring the distance between a focal individual and all other fish in the tank (cm) and time spent within 2 cm of other fish (s).

### Measuring water-borne cortisol

A detailed description of the water-borne hormone extraction protocol, resuspension and dilutions, validations, and enzyme-immuno-assay plate analysis is provided in Appendix A of the Supplementary Material. Final cortisol values (pg/ml) were multiplied by the total resuspension volume (0.720 ml), divided by SL, and multiplied by 2 to obtain cortisol release rates in the unit of pg/mm/h (note that SL is strongly correlated with body mass in 2019:  $R^2 = 0.87$ , N = 182; we do not have mass data from the first year due to a technical issue). The use of cortisol EIA kits to assay water-borne cortisol for the closely related *Gambusia geiseri* had previously been validated by Blake & Gabor (2014) and Blake et al. (2015). Crovo et al. (2015) validated the cortisol kits for *C. venusta*.

### Statistical analyses

We provide a detailed description of our statistical analyses in Appendices B–E of the Supplementary Material (including Tables S2–S5 and Fig. S2–S5). In short, we tested five questions using linear mixed-effects models (LMM) and generalized least squares (GLS) taking into account the non-independence of individuals within the same site and the heterogeneity of variance between sites (Zuur et al. 2009). First, we examined how cortisol release rates varied across treatments (baseline, agitation, and recovery) to test whether the fish in each population showed a stress response to agitation and then a negative feedback. Second, we tested whether four aspects of the GC profile were related to urbanization, expressed as % of developed land in the analyses of *G. affinis* data and as a categorical predictor for *C. venusta* because the latter showed non-linear relationships in diagnostic plots. The dependent variable in each of the four models was baseline cortisol release rate, agitation (stress-induced) cortisol release rate, the magnitude of the stress response expressed as the relative change of cortisol release rate in response to the stress of agitation (stress-induced change):  $100 \times (\text{agitation} - \text{baseline}) / \text{baseline}$ , and the magnitude of negative feedback (for *G. affinis*) expressed as the



**Fig. 2** Cortisol release rates (mean  $\pm$  SE) in baseline, agitation, and recovery treatments along the gradient of urbanization in *Gambusia affinis* and *Cyprinella venusta*. Note that the Y axis has a logarithmic scale. Asterisks above the second and third error bar in each cluster connected by grey lines indicate the significance of the change from baseline to agitation and from agitation to recovery, respectively (\* $0.05 > P > 0.01$ , \*\* $P < 0.001$ ).

relative change from agitation to recovery levels as:  $100 \times (\text{agitation} - \text{recovery}) / \text{agitation}$  (Lattin and Kelly 2020). When testing the relationship between the magnitude of stress response and the intensity of urbanization, we repeated the analysis by excluding a population that did not show a significant cortisol response to agitation; this choice is explained in Appendix B. Third, we tested whether fecundity, total RA, and individual offspring dry mass were related to urbanization. Fourth, we tested whether total RA, a proxy for fitness, was related to baseline cortisol release rate, stress response, and negative feedback in the fish overall, and whether the relationships between RA and GC variables varied across the gradient of urbanization. Finally, we tested whether fish from different sites along the urbanization gradient differed in the latency to enter the novel environment, individual activity (expressed as the scores along the first axis of a principle component analysis that included the time spent moving, distance moved, and velocity), and group shoaling (expressed as the scores along the first axis of a principle component analysis that included the distance between subjects and time spent within 2 cm of other subjects).

## Results

### *Gambusia affinis*

#### Variation in cortisol across land development

*Gambusia affinis* had a significant stress response to agitation, but they did not have a significant recovery overall (Table S6, Fig. 2). All sites of *G. affinis*, except

for the second least urbanized site (1.32%), had a significant stress response, whereas only the most urbanized site (51.3%) showed significant recovery, indicating negative feedback (Table S6, Fig. 2).

There was a marginally significant positive correlation between urbanization and baseline cortisol release rates (Table 1, Fig. 3A), whereas the stress response did not show a significant linear relationship with urbanization (Table 1, Fig. 3B). However, when we excluded the site that did not respond to agitation, there was a significant negative correlation between stress response and urbanization ( $P < 0.001$ ; Table S7, Fig. 3B). Negative feedback increased significantly with urbanization (Table 1, Fig. 3C).

#### Life-history traits

Fecundity of *G. affinis* increased significantly with body mass, and it did so less rapidly in more urbanized habitats (Table 1, Fig. 4A). The smallest females had higher fecundity in more urbanized habitats than in less urbanized habitats, but as females grew the non-urban individuals caught up with their urban conspecifics in fecundity (Fig. 4A). Total reproductive allotment (RA) also increased significantly with body mass, and it did so more rapidly in more urbanized habitats (Table 1, Fig. 4B). The smallest females had similar RA across all habitats, but as females grew the urban individuals had increasingly higher RA than their non-urban conspecifics (Fig. 4B). There was a significant negative relationship between individual offspring dry mass and fecundity, but this relationship

**Table 1** Relationships between glucocorticoid and life-history variables with urbanization (% of developed land), estimated from GLS models for *G. affinis*

Dependent variable	Model coefficients	Estimate	SE	t	P
Baseline cortisol release rate	Intercept	0.068	0.353	0.19	0.847
	Urbanization	0.015	0.008	1.80	0.074
	Date	-0.073	0.030	-2.45	0.016
	Time	-0.001	0.008	-0.15	0.878
Stress response	Intercept	-0.528	0.441	-1.20	0.233
	Urbanization	0.011	0.010	1.09	0.276
	Date	0.083	0.036	2.29	0.024
	Time	-0.008	0.008	-1.03	0.303
Negative feedback	Intercept	-0.374	0.136	-2.76	0.008
	Urbanization	0.017	0.003	5.09	<0.0001
	Date	-0.009	0.011	-0.83	0.409
Fecundity	Intercept	0.950	0.228	4.17	<0.0001
	Female dry mass	0.188	0.015	12.14	<0.0001
	Urbanization	0.028	0.010	2.84	0.005
	Urbanization × Female dry mass	-0.001	0.000	-2.93	0.004
Total reproductive allotment (RA)	Intercept	0.460	0.425	1.08	0.279
	Female dry mass	0.398	0.038	10.47	<0.0001
	Urbanization	-0.034	0.018	-1.95	0.053
	Urbanization × Female dry mass	0.004	0.001	3.17	0.002
Individual offspring dry mass	Intercept	1.516	0.088	17.22	<0.0001
	Fecundity	-0.151	0.031	-4.80	<0.0001
	Urbanization	-0.011	0.004	-2.68	0.008
	Urbanization × Fecundity	0.002	0.001	1.88	0.060

In each model, the first coefficient (intercept) is the estimated mean for zero urbanization, whereas the further coefficients are the slopes of linear relationships with each predictor. Note that several variables were transformed (see Methods), and the model coefficients were not back-transformed to the original scale of the variables. Sample sizes were 149 for baseline cortisol release rate and stress response, 68 for negative feedback, 346 for fecundity, 339 for RA, and 342 for individual offspring dry mass.

did not vary significantly with urbanization (Table 1), although there was a marginally non-significant trend that the relationship was shallowest in the most urbanized sites (Fig. 4C).

#### GC-fitness relationships

For RA across all *G. affinis* sites, the best model identified with forward model selection included a significant interaction between stress response and negative feedback (see Model 7 in Table S3, and Table S4 in Appendix D; Fig. 1C). According to this model, RA increased with increasing negative feedback, but this effect was decreased by increasing stress response (Fig. 1C). Thus, RA was greatest in individuals with high negative feedback and low stress response regardless of the intensity of urbanization (Fig. 1C).

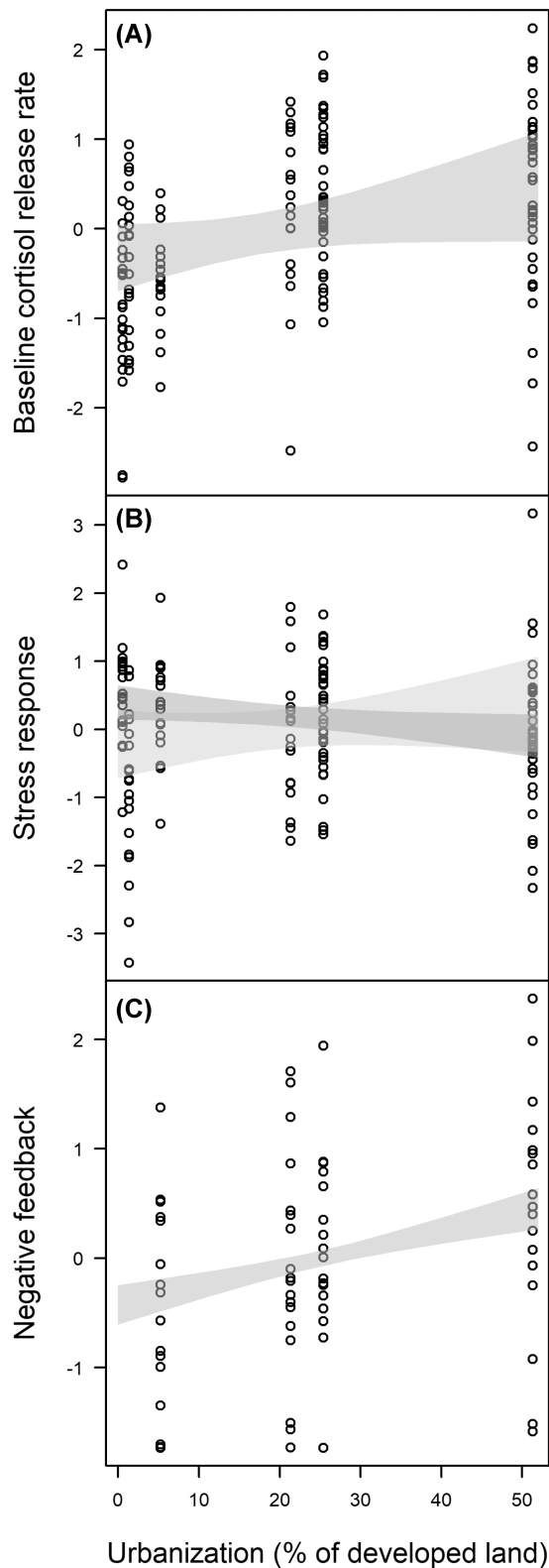
The relationships between RA and GC variables in *G. affinis* did not vary systematically with urbanization: neither the two-way interactions of urbanization with baseline cortisol release rates, stress response, or

negative feedback, nor the three-way interaction of urbanization, stress response, and negative feedback had any significant effect on RA (see Models 11–14 in Table S3, and Table S4 in Appendix D).

#### Behavior

In 2018, neither individual activity (GLS,  $N = 125$ ,  $\chi^2 = 5.64$ ,  $df = 3$ ,  $P = 0.131$ ) nor shoaling (GLS,  $N = 25$ ,  $\chi^2 = 1.30$ ,  $df = 3$ ,  $P = 0.729$ ) differed among the *G. affinis* captured from different habitats. In 2019, latency to emerge from shelter did not vary with habitat of origin (Cox model,  $N = 53$ ,  $\chi^2 = 2.04$ ,  $df = 3$ ,  $P = 0.565$ ), but there was a significant habitat effect on individual activity (GLS,  $N = 45$ ,  $\chi^2 = 13.49$ ,  $df = 3$ ,  $P = 0.004$ ) and on shoaling (GLS,  $N = 26$ ,  $\chi^2 = 36.74$ ,  $df = 3$ ,  $P < 0.001$ ). Specifically, fish from the second-most urbanized site (25.38% developed land) moved less and shoaled more than did the fish from the other three sites (Table S8, Fig. S6).





**Fig. 3** Relationships between urbanization and z-transformed glucocorticoid variables for *G. affinis*. The gray polygons represent the 95% confidence bands of the slopes estimated by the GLS models in Table 1. In panel b, the lighter band is fitted on the entire dataset while the darker band is fitted by excluding the population that did not show significant response to agitation.

### **Cyprinella venusta**

The fish from the four *C. venusta* sites showed a significant cortisol response to agitation (Table S6, Fig. 2). Baseline cortisol release rate differed significantly between habitats (GLS,  $N = 64$ ,  $\chi^2 = 17.29$ ,  $df = 3$ ,  $P < 0.001$ ); it was higher in the fish from the habitat with 1.32% developed land than in fish from the least (0.52%) and most (51.3%) urbanized habitats (Table S9, Fig. 2). Stress response also showed a tendency to differ between sites (GLS,  $N = 64$ ,  $\chi^2 = 6.71$ ,  $df = 3$ ,  $P = 0.082$ ); it was highest in the fish from the habitat with 1.32% developed land and lowest in those from the most urbanized (51.3%) habitat, although all pairwise differences between habitats were non-significant after FDR correction (Table S9).

Reproductive allotment increased with body mass similarly across all habitats (Appendix E: Table S5, Fig. S5). The fish living in the least urbanized site tended to have smaller RA than all the other sites sampled for shiners (Table S9, Fig. 5). Across all shiner sites, RA did not show a significant linear relationship with either baseline cortisol release rates or the magnitude of the stress response (Appendix E: Table S5), and the interaction between urbanization and baseline cortisol release rates was also non-significant (Appendix E: Table S5). However, there was a marginally significant interaction between urbanization and the stress response ( $P = 0.073$ , see Appendix E: Table S5): the relationship between RA and stress response became increasingly negative as urbanization increased (Table S9, Fig. 5).

### **Discussion**

It is not well understood why some species are able to adapt to urban living and others perish (Karr 1981; Wang et al. 2001; Walsh et al. 2005; Shochat et al. 2006; Santangelo et al. 2018; Marques et al. 2019). Studying two tolerant fish species with differing reproductive strategies (i.e., live-bearing and egg-laying), we found that they both exhibited differences in their GC profiles across the urbanization gradient, and that these differences were associated with differences in life-history traits that are major constituents of fitness. Overall, the GC changes observed in more urbanized streams were associated with higher reproductive allotment (RA), suggesting that these endocrine changes were adaptive responses to the urban stream syndrome.

The endocrine mechanisms associated with living in urban habitats were both similar and different between the two species. First, both species showed a tendency toward a reduced GC response to acute stress in more urbanized streams. These trends were not entirely linear, as in each species there was an





the stress response is favored in urban habitats because this allows these tolerant species to realize higher reproductive output. Alternatively, the dampening of the stress response may be a cost rather than an adaptive response (i.e., it may be a physiological consequence of the “wear-and-tear” of frequent stress ([Romero et al. 2009])), which animals may experience in urban habitats, but potentially also in some other habitats like the “outlier” sites in our study.

Furthermore, in *G. affinis*, we found that fitness increased with higher negative feedback, and also that negative feedback increased with urbanization, suggesting that higher reproductive output is facilitated by a further mechanism for keeping the overall GC profile down, and urban populations utilize this existing mechanism for attaining higher RA. Whenever a stress response is mounted (even if a relatively weak one in urban populations), fast negative feedback should be beneficial for reproduction because it minimizes the time the organism is exposed to high GC levels and avoids triggering “emergency” behavioral responses (Partecke et al. 2006; Atwell et al. 2012; Wingfield 2013). Thus, our results suggest that these tolerant fishes cope with urban habitats by upregulating the negative feedback along the HPI axis and perhaps also by suppressing the stress response. Interestingly, this pattern differs from a recent finding on common toad (*Bufo bufo*) tadpoles, where populations in anthropogenic habitats had a higher stress response as well as upregulated negative feedback (Bókony et al. 2021). Altogether, these results suggest that tolerant species may apply partially different endocrine mechanisms for coping with urban habitats, depending on species and/or life-history context such as breeding females versus developing larvae. However, more research will be needed to uncover the sources of GC variability across habitats and to test the robustness of the patterns we found.

We found that baseline cortisol release rates were slightly (but not quite significantly) higher in more urban sites of *G. affinis*, while there was no such pattern in *C. venusta*. Elevated cortisol aids in energy metabolism and maximizing oxygen uptake during low oxygen conditions, which may be more likely in more urbanized streams (McDonald et al. 1991). However, Vitousek et al. (2018) suggested that organisms in demanding environments may benefit from elevating baseline GCs to support energetic regulation only if this elevation is coupled with mounting a relatively weak acute GC response to stress. This may explain the trends we found in *G. affinis* for slightly higher baseline cortisol release rates and slightly lower stress response in more urbanized sites, although the interaction between urbanization, baseline cortisol release

rate and stress response was not significantly associated with RA (Table S4). Another possibility is that high reproductive investment in urban sites may mediate variation in GC profiles rather than *vice versa*, although there is some evidence that reproductive effort may not be a direct driver of GC variation in our case. First, in *G. affinis*, we found no significant effect of gestational stage (developmental stage of the embryo) on baseline cortisol release rates across years and no interaction with urbanization (see Supplementary Material Table S10; Fig S7). Second, Kim et al. (2019) found that cortisol release rates in a laboratory population of *Poecilia latipinna*, another poeciliid fish, did not change with increasing gestational stage. Because later gestational stages are closer to birth and could be more costly and hence stressful for females, the lack of correlation between baseline cortisol release rate and gestational stage suggests that between-individual differences in actual reproductive effort might not have a strong effect on their GC levels. Nevertheless, experimental studies will be needed to ascertain the direction of the relationship between reproductive investment and GC profiles along the gradient of urbanization.

In *G. affinis*, both fecundity and RA was overall higher in more urbanized streams. We also found a similar pattern in *C. venusta*: the least urbanized site had the smallest RA. These findings indicate that, in these two freshwater species, urbanization favors phenotypes with a large investment in current reproduction (Araya-Ajoy et al. 2018). Several aspects of urban stream habitats may contribute to this change in life history, including low predation pressure (Ghalambor et al. 2004), warmer temperatures associated with urban heat islands (Brans et al. 2018b) and wastewater discharges (Vondracek et al. 1988; Byström et al. 2006; Rius et al. 2019), and higher water flow fluctuations (Stearns 1983; Bennett et al. 2016). Interestingly, our results suggesting high RA of aquatic organisms in urban settings is opposite to the general pattern observed in birds, which tend to exhibit reduced brood sizes and reduced offspring size in cities (Sepp et al. 2018). Hence, the particular selective agents driving either life-history strategies in urban environments appear drastically different between terrestrial and aquatic systems. Furthermore, our findings on *G. affinis* also suggest variation across the urbanization gradient in life-history trade-offs. In more urbanized habitats, total RA of *G. affinis* showed a steeper positive relationship with body mass whereas fecundity showed a shallower positive relationship with body mass, meaning that for the same amount of growth the urban fish realized lower increases in fecundity but higher increases in RA compared to non-urban fish. Increases in fecundity

came at a cost of decreased offspring size in non-urban populations but slightly (although not quite significantly) less so in more urbanized populations. These results suggest that the higher food availability of urban streams may change the allocation strategies between major life-history aspects including growth, fecundity, and offspring size, similarly to guppies, *Poecilia reticulata*, another invasive live bearing fish where individuals in urban populations had more food and relaxed life-history trade-offs compared to those in less urban areas (Santana Marques et al. 2020).

In contrast with GCs and life-history traits, behavior showed less variation in *G. affinis* across habitats. In 2018, fish showed no significant differences in activity or shoaling. In 2019, using slightly different methods than in 2018, we found no difference in latency to emerge from shelter across habitats; however, fish from the 25.38% developed habitat moved less and shoaled the most. We would have expected to find less shoaling by fish from more urbanized habitats because shoaling is usually viewed as an antipredatory mechanism (Pitcher et al. 1986; Laland and Williams 1997) and urban streams typically have lower diversity of fish predators (Paul and Meyer 2001). Furthermore, higher investment in current reproduction is often accompanied by risk-prone behaviors, which is another reason why it is surprising that we found no systematic change in the behavior of *G. affinis* across the urbanization gradient. It is possible that gene flow and/or fish movement between streams, or heterogeneity in predation risk across sites (independently of urbanization) may account for the limited variation in behavior across populations. While ample research has been done in urban environments in terrestrial habitats where frequent encounters with humans lead to reduced risk perception and increased boldness (Sepp et al. 2018; Sol et al. 2018), behavioral responses of the freshwater fauna deserve more attention in our pursuit of understanding the intraspecific mechanisms of coping with anthropogenic habitat change.

Our results also contribute to understanding how animals may respond to urbanization by changes along the fast-to-slow pace-of-life continuum (also known as the pace-of-life syndrome or POLS), which is a suite of physiological, behavioral, and reproductive traits that coevolve as adaptations associated with the life-history trade-off between current and future reproduction (Ricklefs and Wikelski 2002; Dammhahn et al. 2018; Montiglio et al. 2018). According to this theory, “fast-living” organisms that prioritize current reproduction over survival through fast body growth rates, early maturity, short lifespans, and a high reproductive effort per breeding attempt, also differ in physiology and risk-taking behavior from “slow-living” individuals that

prioritize survival and future reproduction through slow growth rates, late maturity, long lifespans, and low reproductive effort per breeding attempt (Araya-Ajoy et al. 2018). Accumulating research in birds shows that changes in POLS may be important for adapting to urbanization (Sepp et al. 2018), although in a complex way that is further shaped by cognitive capacity (Sayol et al. 2020) and syndrome break-up due to altered risk perception (Sol et al. 2018). Our present study on stream-living fish supports this complex picture, tentatively suggesting that urbanization might select toward fast life histories for freshwater fishes but without accompanying changes in risk-taking behavior. Although we did not directly test the effects of urban stream syndrome on POLS, this area is a fruitful direction for further research (Brans et al. 2018a; Debecker and Stoks 2019).

Taken together, our findings demonstrate that urbanization alters the stress physiology and life history of two tolerant species of fish, and that their reproductive output may be mediated by variation in GC regulation in response to the environment. These phenotypic changes favor larger reproductive allotment, which may allow for capitalizing on the altered ecological conditions of urban streams. These results inform the mechanisms driving community structure in freshwater associated with land-use converted areas. Further research using common garden experiments is needed to explore whether adaptive phenotypic changes occurred in urban areas via phenotypically plastic responses or genetic changes (Bókony et al. 2021; Lambert et al. 2021).

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## Supplementary data

Supplementary data available at *ICB* online.

## Data availability

Data available on request.

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