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Coping with urban habitats via glucocorticoid regulation: physiology, behavior, and life history in stream fishes

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Abstract 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.

Key-words: cortisol, human-induced environmental change, pace-of-life syndrome, stress physiology, urban stream syndrome

Introduction

Anthropogenic alterations to habitat through land-use conversion contribute significantly to wildlife population extinctions and loss of biodiversity (Brooke Mde et al. 2008; Ceballos et al. 2015; Turner et al. 2007). 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 (Abolins-Abols et al. 2016; Bonier and Martin 2016; Ibanez-Alamo et al. 2020; Sol et al. 2013), 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 (Gabor et al. 2018; Lundberg et al. 2000; Ricciardi and Rasmussen 1999). 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; Meyer et al. 2005; Paul and Meyer 2001; Rahel 2002; 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 Bonier 2012; Fraker et al. 2002; French et al. 2018; Gabor et al. 2018; Sepp et al. 2018; Seress and Liker 2015; Sol et al. 2013).

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; Côte et al. 2021; Kern and Langerhans 2018; Limburg and Schmidt 1990). 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 (Bonier 2012; Dantzer et al. 2014; Ibanez-Alamo et al. 2020; Jeffrey et al. 2015; Ouyang et al. 2019; Partecke et al. 2006). 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 (Guindre-Parker 2018; Romero et al. 2009), 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 (Barton 2002; Romero 2004; Wendelaar Bonga 1997). 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 (Dallman et al. 1992; Sapolsky 1983). 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 (Vitousek et al. 2019; Wingfield 2013). Therefore, the highest fitness may be associated with a robust stress response and fast negative feedback (Figure 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 (Bonier 2012; Partecke et al. 2006). In this case, the highest fitness may be achieved by individuals with the lowest stress response (Figure 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 eutrophicated 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 disproportionally 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 (Frias-Alvarez et al. 2014; Roff 2002; Stearns 1989), and this trade-off may be alleviated in food-rich anthropogenic environments (Santana Marques et al. 2020; Snell-Rood et al. 2015).

Behavioral changes are also often observed in the altered environments of urban habitats, mostly in the form of more risk-prone behaviors (French et al. 2018; Miranda et al. 2013; Sih et al. 2011). Behavioral traits like high activity and exploration may be favored during various stages of urbanization (Polverino et al. 2018; Sih et al. 2012; Sol et al. 2013). 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, 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 (Figure 1a,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 (Meador and Carlisle 2007; Walser and Bart Jr 1999). 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 (Haynes and Cashner 1995; Krumholz 1948). There are significant differences in the size and number of offspring of female *G. affinis* across habitats (Reznick et al. 1990; Stearns 1983).

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 Fish and Wildlife Scientific Permit. We collected fish from six streams located within the Edward's Plateau region of Central Texas (Figure 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 percent of developed land in the subwatershed surrounding each stream sampling site (Table S1), as quantified by the percent 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: Blake et al. 2015; Blake and Gabor 2014; Scott and Ellis 2007) in the field. Within 20 minutes 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 ºC 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 hours at 55 ºC. 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 25ºC and fed them tropical fish flakes (TetraMin) once daily. Following 40-50 hours, we transferred 5 fish into a new container for behavioral observations. In 2018, this was a 37.85 L tank (50.8 cm × 25.4 cm × 30.5 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 mins 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 cm × 9 cm × 18 cm) filled with dechlorinated water and containing a square cutout for a door (5 cm × 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 cm × 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 cm

× 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 × (agitation – baseline) / baseline), and the magnitude of negative feedback (for *G. affinis*) expressed as the relative change from agitation to recovery levels as: 100 × (agitation – recovery) / 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, Figure 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, Figure 2).

There was a marginally significant positive correlation between urbanization and baseline cortisol release rates (Table 1, Figure 3a), whereas the stress response did not show a significant linear relationship with urbanization (Table 1, Figure 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, Figure 3b). Negative feedback increased significantly with urbanization (Table 1, Figure 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, Figure 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 (Figure 4a). Total reproductive allotment (RA) also increased significantly with body mass, and it did so more rapidly in more urbanized habitats (Table 1, Figure 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 (Figure 4b). There was a significant negative relationship between individual offspring dry mass and fecundity, but this relationship 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 (Figure 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; Figure 1c). According to this model, RA increased with increasing negative feedback, but this effect was decreased by increasing stress response (Figure 1c). Thus, RA was greatest in individuals with high negative feedback and low stress response regardless of the intensity of urbanization (Figure 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, χ^2 = 5.64, df = 3, P = 0.131) nor shoaling (GLS, N = 25, χ^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, χ^2 = 2.04, df = 3, P = 0.565), but there was a significant habitat effect on individual activity (GLS, N = 45, χ^2 = 13.49, df = 3, P = 0.004) and on shoaling (GLS, N = 26, χ^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, Figure S6).

Cyprinella venusta

The fish from the four *C. venusta* sites showed a significant cortisol response to agitation (Table S6, Figure 2). Baseline cortisol release rate differed significantly between habitats (GLS, N = 64, χ^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, Figure 2). Stress response also showed a tendency to differ between sites (GLS, N = 64, χ^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, Figure S5). The fish living in the least urbanized site tended to have smaller RA than all the other sites sampled for shiners (Table S9, Figure 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, Figure 5).

Discussion

It is not well understood why some species are able to adapt to urban living and others perish (Karr 1981; Marques et al. 2019; Santangelo et al. 2018; Shochat et al. 2006; Walsh et al. 2005; Wang et al. 2001). 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 "outlier" site that did not mount a significant stress response despite low urbanization (*G. affinis* at the site with 1.32% urbanization) or had much higher cortisol release rates than expected by its low urbanization (*C. venusta* at the site with 0.52% urbanization). Both high GC levels and failure to respond to acute stress may result from chronic

stress and might indicate pathological changes of the HPI axis (Dickens and Romero 2013), although we have no data to explore whether the two "outlier" sites had been exposed to such effects. Among the remaining sites in both species, fish in the more urbanized sites showed relatively weak stress responses, relatively high RA, and a trend toward a negative correlation between stress response and RA. Although the variation in GC profiles may be influenced by several factors, some of which may act independently of urbanization (including idiosyncratic differences between streams in characteristics such as population density or interactions with other species), altogether these findings may suggest that dampening 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 (Atwell et al. 2012; Partecke et al. 2006; 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 (Byström et al. 2006; Rius et al. 2019; Vondracek et al. 1988), and higher water flow fluctuations (Bennett et al. 2016; Stearns 1983). 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 (Laland and Williams 1997; Pitcher et al. 1986) 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

476 attention in our pursuit of understanding the intraspecific mechanisms of coping with
477 anthropogenic habitat change.

478 Our results also contribute to understanding how animals may respond to urbanization by
479 changes along the fast-to-slow pace-of-life continuum (also known as the pace-of-life syndrome
480 or POLS), which is a suite of physiological, behavioral, and reproductive traits that coevolve as
481 adaptations associated with the life-history trade-off between current and future reproduction
482 (Dammhahn et al. 2018; Montiglio et al. 2018; Ricklefs and Wikelski 2002). According to this
483 theory, "fast-living" organisms that prioritize current reproduction over survival through fast
484 body growth rates, early maturity, short lifespans, and a high reproductive effort per breeding
485 attempt, also differ in physiology and risk-taking behavior from "slow-living" individuals that
486 prioritize survival and future reproduction through slow growth rates, late maturity, long
487 lifespans, and low reproductive effort per breeding attempt (Araya-Ajoy et al. 2018).
488 Accumulating research in birds shows that changes in POLS may be important for adapting to
489 urbanization (Sepp et al. 2018), although in a complex way that is further shaped by cognitive
490 capacity (Sayol et al. 2020) and syndrome break-up due to altered risk perception (Sol et al.
491 2018). Our present study on stream-living fish supports this complex picture, tentatively
492 suggesting that urbanization might select toward fast life histories for freshwater fishes but
493 without accompanying changes in risk-taking behavior. Although we did not directly test the
494 effects of urban stream syndrome on POLS, this area is a fruitful direction for further research
495 (Brans et al. 2018a; Debecker and Stoks 2019).

496 Taken together, our findings demonstrate that urbanization alters the stress physiology
497 and life history of two tolerant species of fish, and that their reproductive output may be
498 mediated by variation in GC regulation in response to the environment. These phenotypic

499 changes favor larger reproductive allotment, which may allow for capitalizing on the altered
500 ecological conditions of urban streams. These results inform the mechanisms driving community
501 structure in freshwater associated with land-use converted areas. Further research using common
502 garden experiments is needed to explore whether adaptive phenotypic changes occurred in urban
503 areas via phenotypically plastic responses or genetic changes (Bókony et al. 2021; Lambert et al.
504 2021).
505

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773

774 **Table 1** Relationships between glucocorticoid and life-history variables with urbanization (% of
775 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

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

781 Figure: preference for color: online only.

782

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

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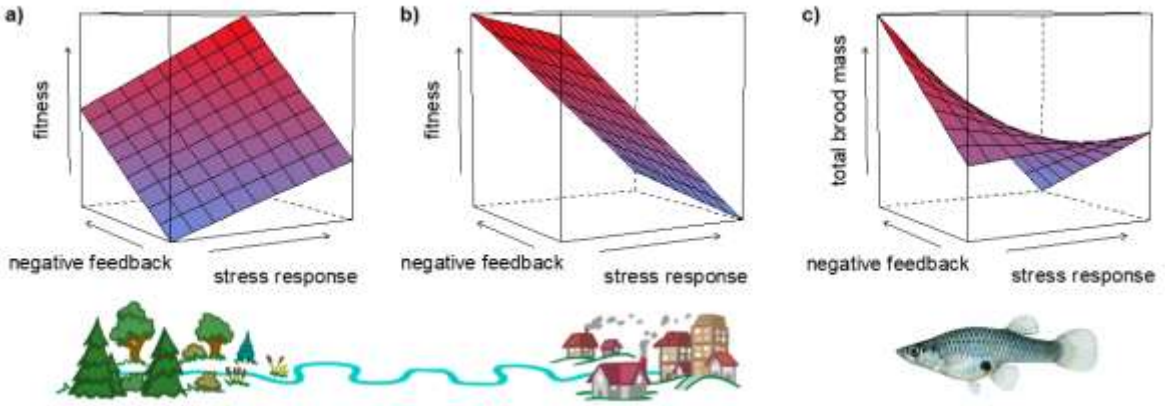
790 [color version for online appearance:]



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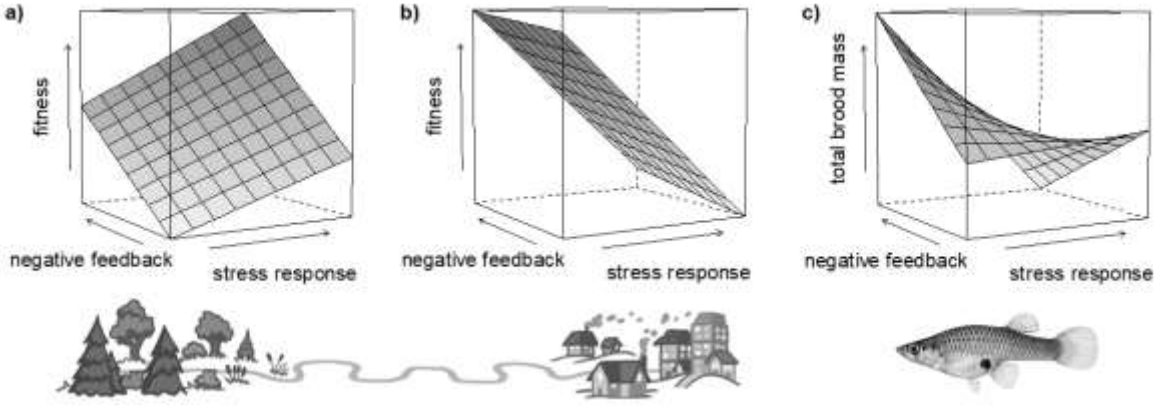

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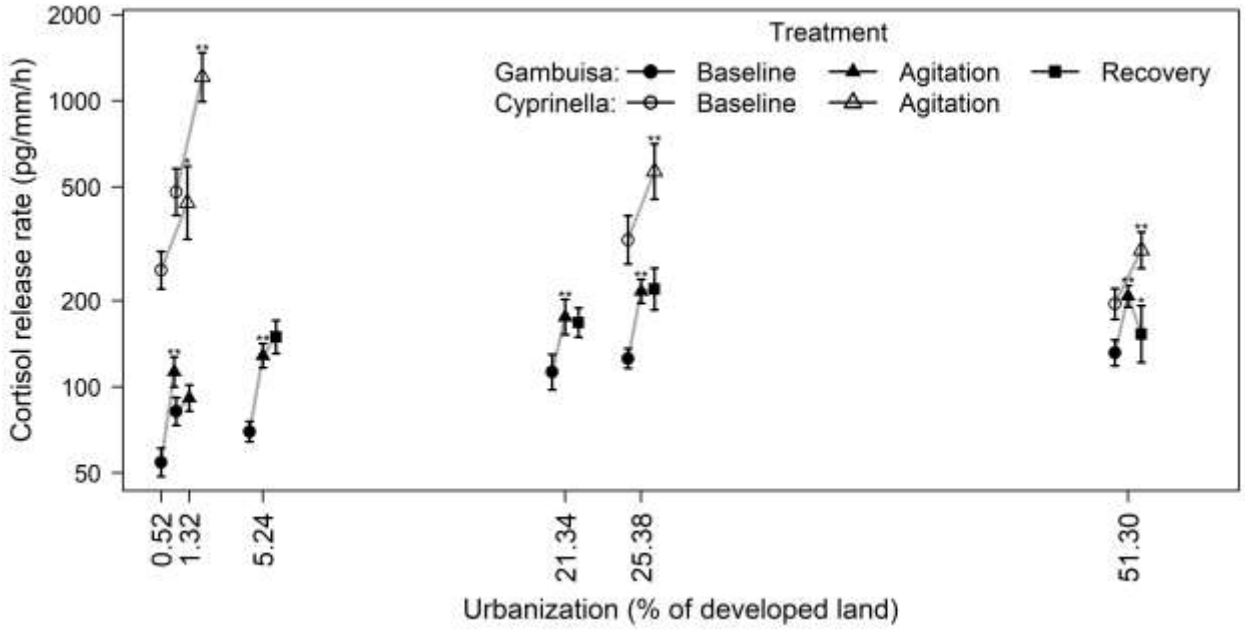
Fig. 2 Cortisol release rates (mean ± SE) in baseline, agitation, and recovery treatments along the gradient of urbanization in *Gambusia affinis* and *Cyprinella venusta*. Note that the Y axis has a logarithmic scale. Asterisks above the second and third error bar in each cluster connected by grey lines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



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

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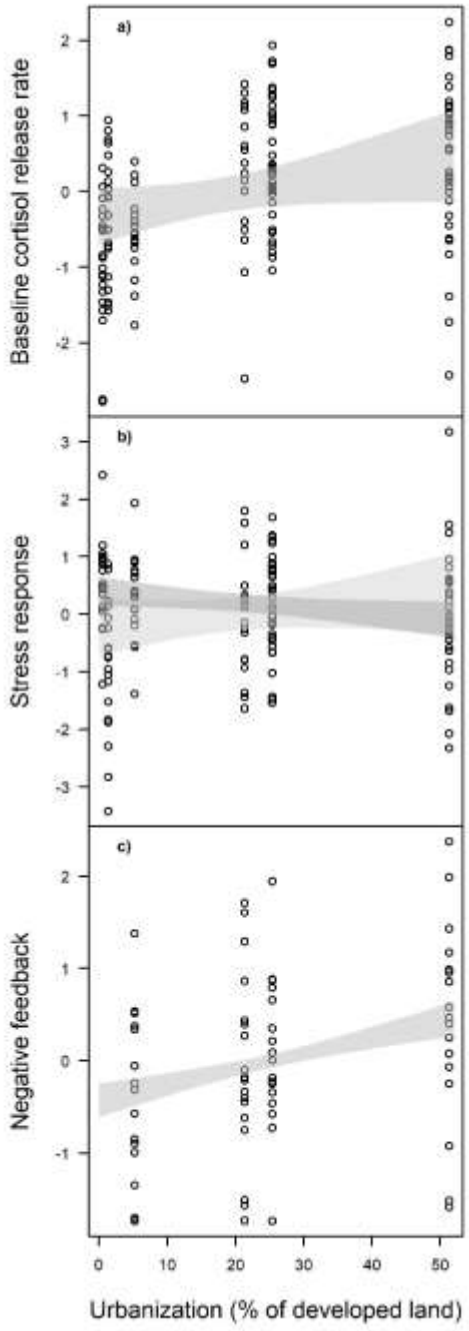

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Fig. 4 Relationships of (a) fecundity (ln number of eggs and embryos) and (b) RA (total dry brood mass; mg^{-2}) with female dry mass (mg^{-2}), and (c) individual offspring dry mass (mg^{-2}) with fecundity for *G. affinis*. Slopes with 95% confidence intervals, predicted from the GLS models in Table 1, are shown for low, medium, and high urbanization levels. These figures represent predicted relationships at 3 points along the (continuous) urbanization gradient.

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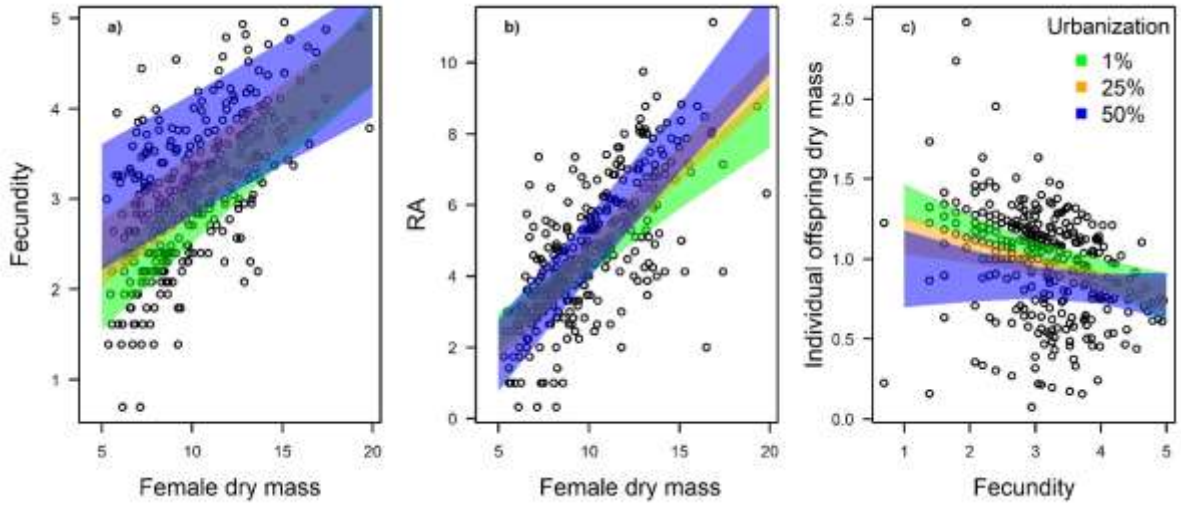

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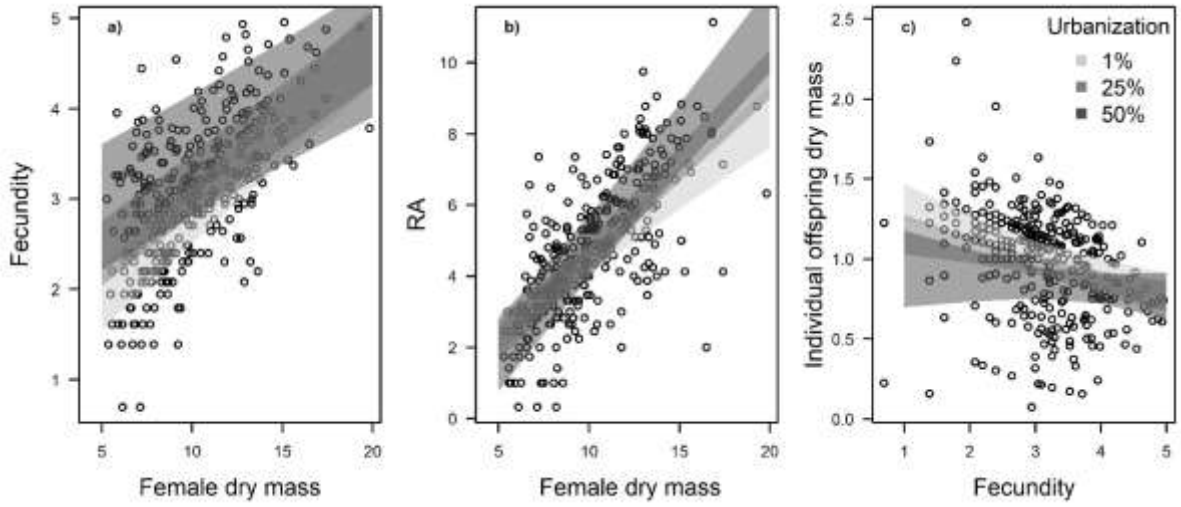

819 **Fig. 5** Relationship between RA and stress response in *C. venusta* in three different habitats along the
820 urbanization gradient. The gray polygons represent the 95% confidence bands of the slopes, predicted for
821 the mean female dry mass for all individuals from the final GLS model in Supporting Information,
822 Appendix E, Table S5.

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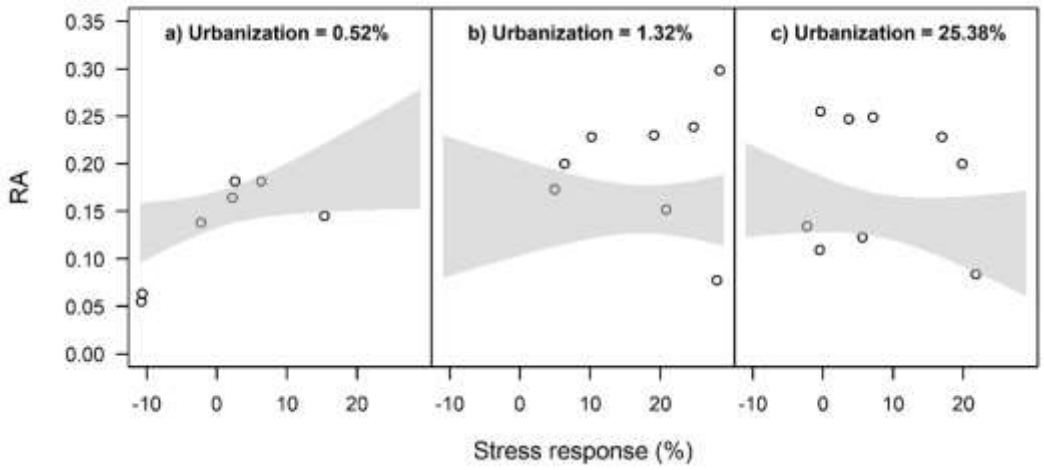

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825

SUPPLEMENTARY INFORMATION

Coping with urban habitats via glucocorticoid regulation: physiology, behavior, and life history of tolerant stream fishes

Table of contents:

852 **Table S10** The effect of gestational stage and percent development on baseline cortisol across
853 years

854 **Fig. S7** The relationship between gestational stage and % development on baseline cortisol
855 across years

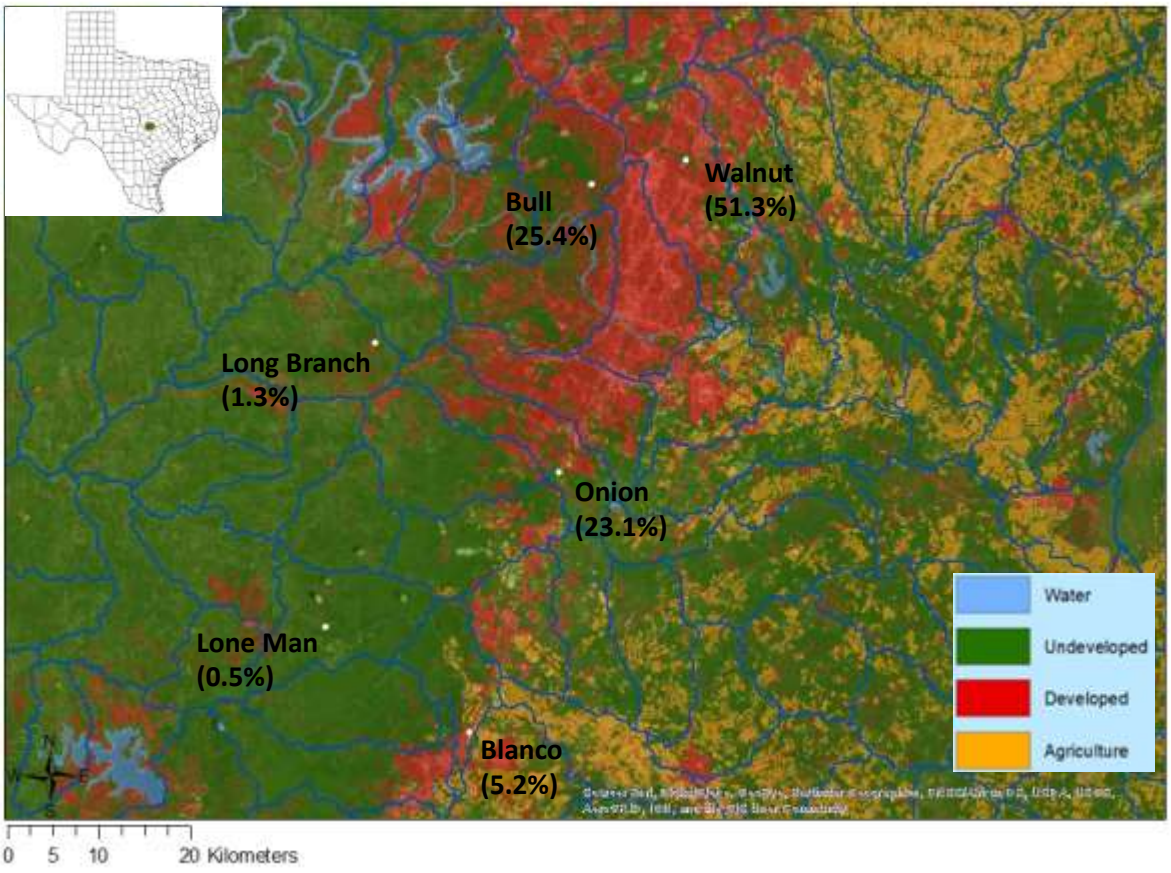
856

857 **Table S1** Description of the sampling sites. Land cover classes are based on the subwatershed area surrounding the sampling site.
858 TDS is total dissolved solids

Year	Developed Land (%)	Undeveloped Land (%)	Agricultural Land (%)	Latitude, longitude	Water T (°C)	pH	Conductivity (µs/cm)	Salinity (ppt)	TDS (g/l)	NO_3
2018	25.38	73.45	0	30.7806, -97.7785	30.8	8.33	583	0.2	0.34	
2019					27.1	7.78	564	0.3	0.35	0.55
2018	51.3	46	2.03	30.39828, -97.6852	24.8	8.20	469	0.2	0.31	
2019					25.3	7.89	446	0.2	0.28	1.16
2018	1.32	98.57	0	30.39828, -98.0400	26.4	8.12	388	0.4	0.52	
2018	0.52	98.46	0	30.39828, -97.9906	27.7	8.10	561	0.3	0.35	
2019	21.34	77.98	0.4	30.1328, -97.8106	29.8	7.71	595	0.3	0.35	0.82
2018	5.24	82.78	10.95	29.9120, -97.8982	29.9	7.80	424	0.2	0.26	
2019					24.6	7.81	437	0.2	0.29	0.45

859

860 **Fig. S1** Map of the six study areas in Central Texas, USA with their percent developed land
861 cover in parenthesis
862



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Appendix A: Measuring water-borne cortisol

We passed individual water samples through C18 Solid Phase Extraction (SPE) columns (SepPak Vac 3 cc/500 mg; Waters Inc.) primed with 4 ml methanol and 4 ml distilled water. We extracted the hormones with 4 ml methanol into borosilicate glass tubes via a vacuum manifold at a pressure of 20 mmHg. We dried eluted samples in a 37 °C water bath using nitrogen gas flowing through an Evap-O-Rac (Cole-Parmer Inc.). Each dried sample was then resuspended in a mixture of 5% ethanol (95% lab grade) and 95% enzyme-immunoassay (EIA) buffer (Cayman Chemical Company Inc.) to a final volume of 720 µl, and vortexed for 2 hours. Samples were then diluted (1:20 for *G. affinis*; 1:100 for *C. venusta*) in EIA buffer and run in duplicate on Cortisol EIA plates (Cayman Chemical Company Inc., No 500360). Absorbance was read using a spectrophotometer plate reader (ELX 800; Biotek Instruments Inc.) set to 405 nm.

We used a pooled sample of cortisol from non-experimental fish as our control in quadruplicate on each of the 11 experimental plates for *G. affinis* and 5 plates for *C. venusta*. The cortisol assays have a range from 6.6 to 400 pg/ml and a sensitivity of approximately 35 pg/ml. For *G. affinis* our inter-assay coefficient of variation for the control sample was 11.96% and our intra-assay coefficients of variation ranged from 0.70% to 10.99%. For *C. venusta* our inter-assay coefficient of variation for the control sample was 14.36% and our intra-assay coefficients of variation ranged from 0.34% to 8.91%.

Appendix B: Statistical analyses

All statistical analyses were done using R version 3.6.3 (R Core Team 2020). During each analysis, we examined the data and model residuals graphically and chose the type of model (as detailed below) and transformation that ensured that the distribution of residuals conformed to the assumptions of our models. For *G. affinis*, all cortisol release rates and fecundity were natural log-transformed, and female dry mass, RA, and individual offspring dry mass were square-root transformed. To quantify the magnitude of stress response, we calculated the relative change of cortisol release rate in response to the stress of agitation (stress-induced change) as: 100 × (agitation – baseline) / baseline (Bókony et al. 2021). Because this variable was strongly right-skewed, we log-transformed it (after adding 54 to avoid negative values). There is no consensus in the current literature whether the transcriptomic, phenotypic, and fitness effects of stress are better predicted by the stress-induced levels of glucocorticoids or by their stress-induced increase (Vitousek et al. 2018). All else being equal, a higher absolute GC concentration has stronger effects (Romero 2004); however, the effects of GCs depend on other regulators such as the abundance of GC receptors, corticosteroid binding globulins, and enzymes that metabolize GCs (Breuner et al. 2003; Lattin and Kelly 2020). For example, long-term elevation of baseline GCs can be accompanied by decreased receptor production and thus diminished biological effects at a given GC concentration (Romero, 2004). Therefore, the increase from baseline to acute stress-induced GC levels might better express the strength of the stress response when organisms differ in their baseline levels (Vitousek et al. 2018). Following this logic, we preferred to use the stress-induced change, rather than the absolute stress-induced values, as proxy for stress response, because our data showed a trend that *G. affinis* differ in their baseline values along the urbanization gradient (see Table 1 in main text), and there was strong correlation between baseline and stress-induced cortisol release rates ($r = 0.73$, $P < 0.001$, $N = 149$). We chose to quantify the stress-induced increase as relative change (rather than absolute difference) to be consistent with the calculation of negative feedback, for which relative change was proposed to be the best metric (Lattin and Kelly 2019). Accordingly, we quantified negative feedback as the relative change from agitation to recovery levels as: 100 × (agitation – recovery) / agitation (Lattin and Kelly 2019). This variable was strongly left-skewed, so we transformed it to the 3^{rd} power adding 192 to avoid negative values. As cortisol and life-history variables were measured

915 the same way in the two years, we analyzed these data by pooling the two years. This approach
916 was justified by preliminary analyses using only those two sites sampled in both years, which
917 showed that cortisol levels and the relationships between RA or fecundity and female dry mass
918 were similar between the two years (Appendix C: Figure S2–S4). As the behavioral tests differed
919 between years, the behavioral data were analyzed separately for the two years. Throughout all
920 analyses, we used residual plots to check that our models met the assumptions of linearity,
921 normality, and homogeneity of variances, and we calculated the variance inflation factor (VIF) to
922 check multi-collinearity in multi-predictor models. Three influential outliers that we identified in
923 the relationship between RA and female dry mass (Appendix C: Figure S4) were excluded from
924 the analyses of RA. In all analyses, we allowed the within-site variance to differ between sites
925 (using the "varIdent" function) because diagnostic plots indicated heteroscedasticity.
926 First, we examined how cortisol release rates varied across treatments (baseline,
927 agitation, and recovery) to test whether the fish showed a stress response to agitation and then a
928 negative feedback regardless of habitat. Since this is a within-subject question, we used a linear
929 mixed-effects model (LMM) ("lme" function in the "nlme" package) with two random factors,
930 individual fish nested within sites, to account for repeated measures. This model tested the
931 overall response of GCs to agitation and recovery across all fish. Then we tested the same
932 question for each site, by using LMM with individual as the only random factor, including site as
933 a fixed factor and its interaction with treatment, and extracting site-specific comparisons of
934 estimated marginal means ("emmeans" function in the "emmeans" package). P-values from
935 multiple comparisons among sites were corrected for type-1 error inflation using the false
936 discovery rate (FDR) method (Pike 2011).
937 Second, we tested whether any aspect of the GC profile was related to urbanization. Since
938 this is a between-subjects (site-averaging) question, we used Generalized Least Squares (GLS)
939 models ("gls" function in the "nlme" package), taking into account the non-independence of
940 individuals within the same site by using the compound symmetry correlation structure (Zuur et
941 al. 2009). The dependent variable in each of the three models was baseline cortisol release rate,
942 the magnitude of the stress response, and the magnitude of negative feedback. We used
943 urbanization (% of developed land) as a numeric predictor. For the dependent variables measured
944 in both years, we also included capture date (number of days since the first capture day of each
945 year; range: 0-12 days) and time of day (number of minutes from the earliest time of the first

collection time for each year; range: 0-45 min) as covariates into the models. For negative feedback which was measured only in the second year, we could only add date into the model because including the time of day along with urbanization would have led to high multi-collinearity (VIF > 2). Examination of diagnostic graphs showed no clear non-linear patterns in the data. To facilitate the comparison of effect sizes between the three aspects of GC regulation (baseline cortisol release rate, stress induced change, and negative feedback), we z-transformed the three GC variables (mean-centered and divided by standard deviation).

We applied the same GLS approach to examine differences in fecundity, total RA, and individual offspring dry mass across the gradient of urbanization. Note that, although fecundity was a count variable, it did not fit the Poisson distribution; instead, it showed good fit to normal distribution (after log-transformation). Models for fecundity and total RA included female dry mass as a covariate to account for variation in body size, and its interaction with urbanization. The model for individual offspring dry mass included fecundity as a covariate and its interaction with urbanization to investigate how urbanization influenced the trade-off between fecundity and individual offspring dry mass.

Then we tested whether total RA, a proxy for fitness, was related to aspects of the GC profile in the fish overall. We used a forward stepwise model-selection procedure, starting with the three simplest GLS models that included the interaction of female dry mass and urbanization (because in the previous analyses we found that this interaction had a significant effect on RA; see Results), and either baseline cortisol release rate or stress response or negative feedback (all three GC variables were z-transformed before model selection). We increased model complexity to identify the combination of GC variables and their interactions that best explained individual variation in RA (see Appendix D for a detailed description of the model-selection steps). Finally, we used the best GLS model selected by this procedure and continued the forward model selection to test whether the relationship between RA and components of GC regulation varied across the gradient of urbanization (see each step described in Appendix D). We preferred the forward stepwise approach instead of information-theoretic model selection because the former does not require that all models are run with the same dataset, thus allowing more power in our case due to the missing data for negative feedback. Nevertheless, we also present a full model containing all main effects and interactions for which we had testable predictions (see Table S4 in Appendix D).

To analyze behavioral differences along the urbanization gradient in each year, we used urbanization as categorical predictor because diagnostic plots indicated non-linear relationships. We tested the overall effect of urbanization by type-2 analysis-of-deviance tables, and for pairwise comparisons between habitats we used the same methods as above (estimated marginal means with FDR correction). For the latency to enter the novel environment, we used a Cox's proportional hazards model, treating the individuals that did not emerge within 5 minutes as censored observations. For individual activity and group shoaling, we used GLS models. Individual activity was expressed as the scores along the first axis of a principle component analysis (PCA) that included the time spent moving (loading: 0.56), distance moved (loading: 0.61), and velocity (loading: 0.57), and explained 86% of total variance. Shoaling behavior was expressed as the scores along the first axis of a PCA that included the distance between subjects (loading: -0.71) and time spent within 2 cm of other subjects (loading: 0.71), and explained 94% of total variance.

The *C. venusta* data were analyzed with the same approaches as described above for the *G. affinis* data, with the following differences. Because diagnostic plots indicated non-linear relationships with urbanization, we used the % of developed land as a categorical predictor. Stress response, calculated as the relative change (%) from baseline to agitation treatment, required no further transformation. As proxy for RA, we used square-root transformed dry ovary mass, and we omitted a single outlier that had much smaller ovaries than expected by its body mass and violated the model assumptions (Appendix E: Figure S5). The steps of the forward model selection are described in Appendix E.

References not cited in the main text:

Breuner C.W., Orchinik M., Hahn T.P., Meddle S.L., Moore I.T., Owen-Ashley N.T., Sperry T.S., Wingfield J.C., 2003. Differential mechanisms for regulation of the stress response across latitudinal gradients. Am. J. Physiol. - Regul. Integr. Comp. Physiol. 285:1–3.

Lattin C.R., Kelly T.R., 2020. Glucocorticoid negative feedback as a potential mediator of trade-offs between reproduction and survival. Gen. Comp. Endocrinol. 286:113301.

Pike N. 2011. Using false discovery rates for multiple comparisons in ecology and evolution. Methods in Ecology and Evolution. 2(3):278-282.

Appendix C: Comparisons of *G. affinis* data between 2018 and 2019

Two populations (Bull Creek, 25.38%, and Walnut Creek, 51.3%) were sampled in both years. Restricting the dataset to these two populations, we found no significant year effects either on cortisol release rates (Table S2, Fig. S2) or on the relationships of fecundity and RA with body mass (Table S2, Fig. S3-4). This indicates that year effects are unlikely to have caused differences between habitats sampled in different years; thus, the two years' data can be analyzed as a single, pooled dataset.

Fig. S2 Cortisol release rates of *G. affinis* in Bull Creek (25.38%) and Walnut Creek (51.3%) in 2018 and 2019. In each boxplot, the thick middle line and the box, respectively, show the median and interquartile range, and whiskers extend to the most extreme data points within 1.5 × interquartile range from the box

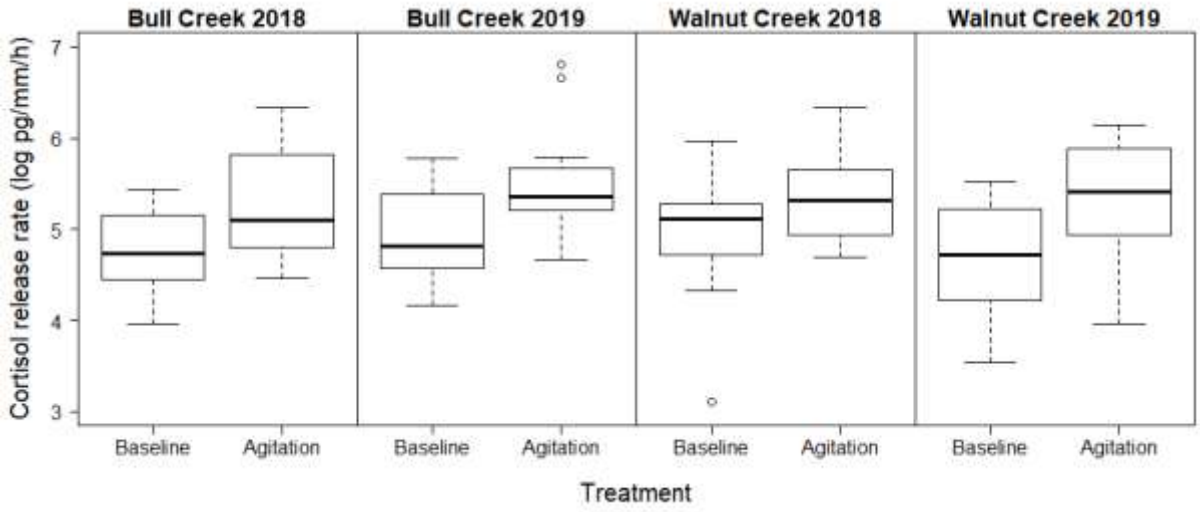

Fig. S3 Relationship between fecundity and dry eviscerated mass of *G. affinis* in Bull Creek and Walnut Creek in 2018 and 2019

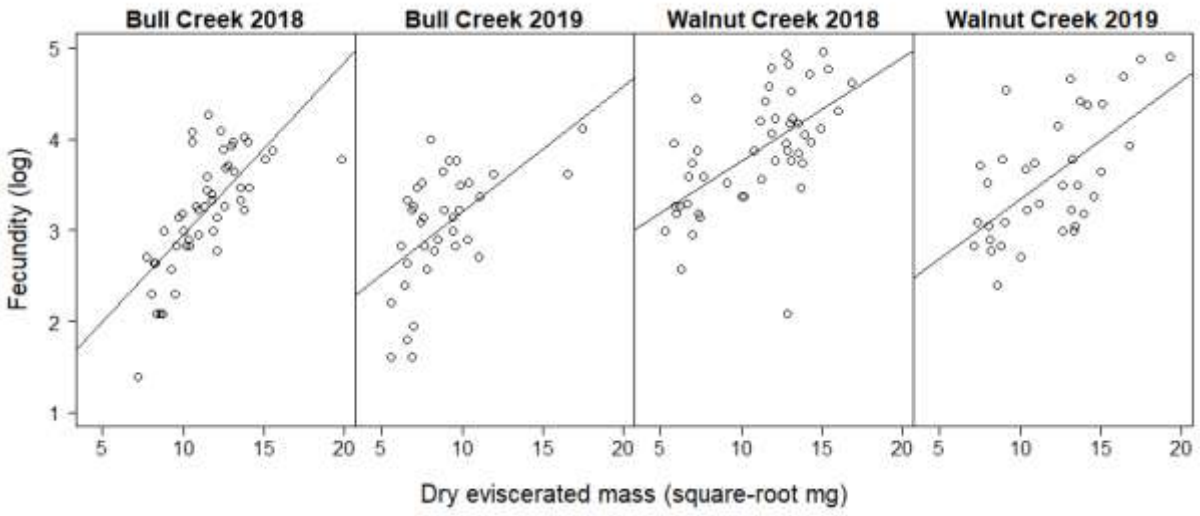

Fig. S4 Relationship between RA (total brood mass) and dry eviscerated mass of *G. affinis* in Bull Creek and Walnut Creek in 2018 and 2019. Three outliers are shown with filled circles; the regression lines were fitted by excluding the outliers

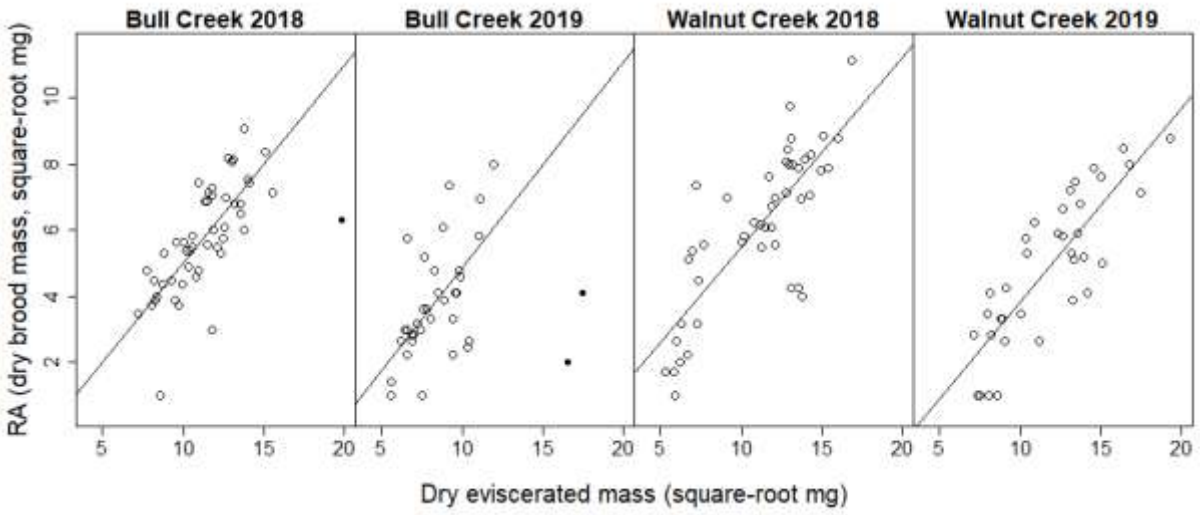

Table S2 GLS models of cortisol release rates (N=148), fecundity (N=170), and RA (N=164) of *G. affinis* in Bull Creek and Walnut Creek. Model terms that include year effects on relevant aspects of the data are highlighted in bold

Dependent variable	Model coefficients	Estimate	SE	t	P
Cortisol release rate	Intercept	4.750	0.120	39.70	<0.0001
	Treatment(agitation)	0.510	0.169	3.00	0.003
	Population(Walnut)	0.250	0.174	1.40	0.154
	Year(2019)	**0.180**	**0.176**	**1.00**	**0.303**
	Treatment(agitation) × Population(Walnut)	-0.170	0.246	-0.70	0.494
	Treatment(agitation) × Year(2019)	**0.060**	**0.249**	**0.20**	**0.810**
	Population(Walnut) × Year(2019)	**-0.440**	**0.262**	**-1.70**	**0.092**
	Treatment(agitation) × Population(Walnut) × Year(2019)	**0.180**	**0.370**	**0.50**	**0.622**
Fecundity	Intercept	1.045	0.312	3.35	0.001
	Dry Eviscerated Mass	0.191	0.027	7.09	<0.0001
	Year(2019)	0.779	0.442	1.76	0.080
	Population(Walnut)	1.576	0.407	3.87	<0.0001
	Mass × Year(2019)	**-0.053**	**0.044**	**-1.21**	**0.230**
	Mass × Population(Walnut)	-0.076	0.035	-2.16	0.032
	Year(2019) × Population(Walnut)	-1.372	0.620	-2.21	0.028
	Mass × Year(2019) × Population(Walnut)	**0.070**	**0.057**	**1.22**	**0.223**
RA	Intercept	-0.965	0.855	-1.13	0.261
	Dry Eviscerated Mass	0.599	0.075	7.97	<0.0001
	Year(2019)	-0.395	1.468	-0.27	0.788
	Population(Walnut)	0.712	1.149	0.62	0.536
	Mass × Year(2019)	**0.024**	**0.161**	**0.15**	**0.883**
	Mass × Population(Walnut)	-0.025	0.100	-0.25	0.804
	Year(2019) × Population(Walnut)	-1.356	1.840	-0.74	0.462
	Mass × Year(2019) × Population(Walnut)	**-0.013**	**0.186**	**-0.07**	**0.945**

The intercept refers to fish in Bull Creek in 2018 and, for cortisol, the baseline sample. "Mass" stands for dry eviscerated mass. For RA, the model excludes three outliers that violated the assumptions of linearity, normality and homoscedasticity (see Fig. S3). Note that several variables were transformed (see Methods), and the model coefficients were not back-transformed to the original scale of the variables

Appendix D: Steps of the forward model-selection procedure for *G. affinis*

The aim of the first part of the model-selection procedure was to find the combination of baseline cortisol release rate, stress response, and negative feedback that best explains the inter-individual variation in RA across all fish (regardless of urbanization). Our "null model" included the interaction of urbanization × dry eviscerated mass (see Table 1 in main text).

- Step 1: Into the null model we added either baseline cortisol release rate or stress response or negative feedback (Models 1-3 in Table S3), and retained the one with the lowest (significant) P-value, which was the stress response (Model 2).
- Step 2: Into the model with stress response, we added either baseline cortisol release rate or negative feedback (Models 4-5 in Table S3). Neither was significant, so we moved on to adding interactions next.
- Step 3: The interaction of stress response was significant with negative feedback but not with baseline cortisol release rate (Models 6-7 in Table S3), so we retained the former.
- Step 4: Into the model that contained the interaction of stress response and negative feedback, we added baseline cortisol release rate either as a main effect or in interaction (Models 8-10 in Table S3). As neither was significant, all these more complex models were discarded.
- Step 5: Therefore, we selected Model 7 as the best model (Table S3).

The aim of the second part of the model-selection procedure was to evaluate whether the relationships between RA and GC variables vary with urbanization. We started with Model 7 that was the best model in the previous model selection.

- Step 6: Into the model that contained the interaction of stress response and negative feedback, we added the two-way interaction between urbanization and either baseline cortisol release rate or stress response or negative feedback (Models 11-13 in Table S2). None of these interactions were significant.
- Step 7: We added the three-way interaction between urbanization, stress response and negative feedback (Model 14 in Table S3); it was non-significant.
- Step 8: Therefore, the best model was still Model 7: no interaction between urbanization and GC variables (Table S3).

There was no significant relationship between female gestational stage and cortisol release rates in 2018 (GLM: population x gestation stage: $F_{3,3} = 0.95$, $p = 0.418$) or 2019 (GLM: population x gestation stage: $F_{3,3} = 1.85$, $p = 0.149$).

As an alternative to the forward stepwise model selection, we also evaluated a full model that included all main effects and interactions for which we had testable predictions. Specifically, the model included the interaction of urbanization × dry eviscerated mass (to allow for different scaling of RA with body size along the urbanization gradient), all three GC variables (baseline cortisol release rate, stress response, and negative feedback), the interaction of baseline × stress response (allowing for the magnitude of stress response to depend on the baseline levels; see Vitousek et al. 2018),the interaction of stress response × negative feedback (allowing for the magnitude of negative feedback to depend on the stress response; see Bókony et al. 2021), and the interactions of urbanization with all GC variables and GC interactions (to allow for different GC regulation along the urbanization gradient). This model was limited to the 58 individuals caught in 2019 due to the missing measurements of negative feedback from 2018. Qualitatively, this model yielded the same results as the forward stepwise model selection (Table S4).

Table S3 GLS models of RA evaluated during forward model selection for *G. affinis*. The relevant term evaluated in each step is highlighted in bold

Model #	Model coefficients	Estimate	SE	t	P
Model 1	Intercept	1.064	0.560	1.90	0.060
	Mass	0.372	0.052	7.11	<0.0001
	Urbanization	-0.080	0.025	-3.24	0.002
	Mass × Urbanization	0.007	0.002	3.37	0.001
	BaselineCort	**0.304**	**0.122**	**2.50**	**0.014**
Model 2	Intercept	0.619	0.538	1.15	0.252
	Mass	0.397	0.052	7.63	<0.0001
	Urbanization	-0.071	0.024	-2.96	0.004
	Mass × Urbanization	0.007	0.002	3.36	0.001
	StressResponse	**-0.262**	**0.101**	**-2.61**	**0.010**
Model 3	Intercept	0.879	0.821	1.07	0.289
	Mass	0.399	0.082	4.86	<0.0001
	Urbanization	-0.047	0.040	-1.18	0.241
	Mass × Urbanization	0.003	0.004	0.81	0.424
	NegativeFeedback	**0.044**	**0.173**	**0.25**	**0.801**
Model 4	Intercept	-0.278	0.456	-0.61	0.543
	Mass	0.501	0.041	12.20	<0.0001
	Urbanization	-0.002	0.010	-0.22	0.824
	Mass × Urbanization	0.007	0.002	3.45	0.001
	StressResponse	-0.175	0.113	-1.55	0.123
	BaselineCort	**0.198**	**0.139**	**1.42**	**0.157**
Model 5	Intercept	0.175	0.469	0.37	0.710
	Mass	0.495	0.052	9.43	<0.0001
	Urbanization	-0.022	0.006	-3.49	0.001
	Mass × Urbanization	0.004	0.004	0.92	0.362
	StressResponse	-0.486	0.202	-2.41	0.020
	NegativeFeedback	**0.209**	**0.179**	**1.17**	**0.248**
Model 6	Intercept	0.904	0.572	1.58	0.116
	Mass	0.381	0.054	7.12	<0.0001
	Urbanization	-0.078	0.024	-3.22	0.002
	StressResponse	-0.184	0.110	-1.67	0.098
	BaselineCort	0.187	0.141	1.32	0.189
	Mass × Urbanization	0.007	0.002	3.46	0.001
	StressResponse × BaselineCort	**0.057**	**0.087**	**0.66**	**0.513**
Model 7	Intercept	1.292	0.808	1.60	0.116
	Mass	0.400	0.081	4.91	<0.0001

	Urbanization	-0.057	0.038	-1.52	0.136
	StressResponse	-0.692	0.208	-3.32	0.002
	NegativeFeedback	0.262	0.169	1.55	0.128
	Mass × Urbanization	0.003	0.004	0.87	0.388
	StressResponse × NegativeFeedback	**-0.487**	**0.196**	**-2.49**	**0.016**
Model 8	Intercept	1.210	0.861	1.41	0.166
	Mass	0.408	0.084	4.83	<0.0001
	Urbanization	-0.057	0.041	-1.41	0.165
	BaselineCort	**-0.032**	**0.195**	**-0.16**	**0.870**
	StressResponse	-0.705	0.227	-3.10	0.003
	NegativeFeedback	0.287	0.181	1.58	0.120
	Mass × Urbanization	0.003	0.004	0.86	0.393
	StressResponse × NegativeFeedback	-0.481	0.197	-2.44	0.018
Model 9	Intercept	1.169	0.876	1.33	0.189
	Mass	0.414	0.086	4.80	<0.0001
	Urbanization	-0.057	0.043	-1.31	0.197
	BaselineCort	-0.052	0.210	-0.25	0.805
	StressResponse	-0.698	0.229	-3.04	0.004
	NegativeFeedback	0.307	0.186	1.65	0.105
	Mass × Urbanization	0.003	0.004	0.80	0.427
	StressResponse × NegativeFeedback	-0.496	0.206	-2.40	0.020
	BaselineCort × StressResponse	**0.070**	**0.189**	**0.37**	**0.713**
Model 10	Intercept	1.880	0.887	2.12	0.039
	Mass	0.337	0.088	3.84	0.000
	Urbanization	-0.105	0.049	-2.17	0.035
	BaselineCort	0.077	0.204	0.38	0.708
	StressResponse	-0.665	0.225	-2.96	0.005
	NegativeFeedback	0.413	0.178	2.32	0.025
	Mass × Urbanization	0.009	0.005	1.78	0.081
	BaselineCort × StressResponse	0.161	0.208	0.78	0.441
	BaselineCort × NegativeFeedback	0.529	0.259	2.04	0.047
	StressResponse × NegativeFeedback	-0.364	0.201	-1.81	0.076
	BaselineCort × StressResponse × NegativeFeedback	**0.281**	**0.300**	**0.94**	**0.354**
Model 11	Intercept	1.317	0.908	1.45	0.153
	Mass	0.400	0.087	4.58	<0.0001
	Urbanization	-0.067	0.048	-1.41	0.166
	BaselineCort	0.067	0.300	0.22	0.826
	StressResponse	-0.716	0.228	-3.14	0.003
	NegativeFeedback	0.283	0.184	1.54	0.131

	Mass × Urbanization	0.005	0.005	0.94	0.353
	StressResponse × NegativeFeedback	-0.508	0.202	-2.51	0.015
	Urbanization × BaselineCort	**-0.007**	**0.014**	**-0.46**	**0.646**
Model 12	Intercept	1.270	0.819	1.55	0.127
	Mass	0.421	0.083	5.08	<0.0001
	Urbanization	-0.062	0.040	-1.57	0.124
	StressResponse	-1.143	0.415	-2.75	0.008
	NegativeFeedback	0.335	0.179	1.87	0.067
	Mass × Urbanization	0.003	0.004	0.80	0.430
	StressResponse × NegativeFeedback	-0.622	0.224	-2.78	0.008
	Urbanization × StressResponse	**0.024**	**0.018**	**1.30**	**0.199**
Model 13	Intercept	1.150	0.936	1.23	0.225
	Mass	0.416	0.098	4.25	0.000
	Urbanization	-0.051	0.041	-1.25	0.217
	StressResponse	-0.694	0.210	-3.30	0.002
	NegativeFeedback	0.334	0.279	1.20	0.236
	Mass × Urbanization	0.003	0.004	0.63	0.534
	StressResponse × NegativeFeedback	-0.479	0.200	-2.39	0.021
	Urbanization × NegativeFeedback	**-0.004**	**0.012**	**-0.36**	**0.718**
Model 14	Intercept	1.437	1.046	1.37	0.176
	Urbanization	-0.080	0.051	-1.57	0.122
	Mass	0.420	0.108	3.90	0.000
	StressResponse	-1.389	0.468	-2.97	0.005
	NegativeFeedback	0.467	0.303	1.54	0.129
	StressResponse × NegativeFeedback	-0.995	0.414	-2.40	0.020
	Urbanization × Mass	0.005	0.005	0.89	0.376
	Urbanization × StressResponse	0.032	0.020	1.63	0.109
	Urbanization × NegativeFeedback	-0.006	0.012	-0.52	0.606
	Urbanization × StressResponse × NegativeFeedback	**0.021**	**0.019**	**1.16**	**0.254**

The models exclude three outliers that violated the assumptions of linearity, normality and homoscedasticity (see Fig. S3). "Mass" stands for dry eviscerated mass. Note that several variables were transformed (see Methods), and the model coefficients were not back-transformed to the original scale of the variables. Baseline cortisol release rates, stress response, and negative feedback were z-transformed for these analyses. Sample size was N = 58 in all models that included negative feedback, and N = 134 in all other models

Table S4 Full GLS model simultaneously testing the relationships of RA with urbanization, GC variables, and their interactions in *G. affinis*. Significant terms are highlighted in bold

Model coefficients	Estimate	SE	t	P
Intercept	1.200	1.275	0.94	0.352
Urbanization	-0.064	0.064	-1.00	0.323
Mass	**0.443**	**0.129**	**3.44**	**0.001**
BaselineCort	-0.214	0.409	-0.52	0.603
StressResponse	**-1.484**	**0.580**	**-2.56**	**0.014**
NegativeFeedback	0.586	0.382	1.53	0.132
Mass × Urbanization	0.003	0.006	0.50	0.617
BaselineCort × StressResponse	0.146	0.559	0.26	0.796
StressResponse × NegativeFeedback	**-1.112**	**0.489**	**-2.27**	**0.028**
Urbanization × BaselineCort	0.002	0.017	0.11	0.914
Urbanization × StressResponse	0.032	0.023	1.40	0.168
Urbanization × NegativeFeedback	-0.011	0.015	-0.75	0.456
Urbanization × BaselineCort × StressResponse	-0.002	0.024	-0.07	0.942
Urbanization × StressResponse × NegativeFeedback	0.026	0.021	1.23	0.225

Appendix E: Steps of the forward model-selection procedure for *C. venusta*.

Our first step was to find the appropriate "null model": the overall relationship between RA (dry ovary mass) and body size (dry eviscerated mass).

- Step 1: We tested the interaction between body size and urbanization, and found it non-significant (Model 1 in Table S5; Fig. S5). Therefore, in our "null model" we retained the main effects of body size and urbanization, without their interaction (Model 2 in Table S5).
- Step 2: Into the "null model", we added either baseline cortisol release rate or stress response; neither was significant (Models 3-4 in Table S5).
- Step 3: We also tested the interaction between baseline cortisol release rate and stress response, which was also non-significant (Model 5 in Table S5).
- Step 4: Into the "null model", we added an interaction between urbanization and either baseline cortisol release rate or stress response (Models 6-7 in Table S5). Because neither of these interactions was significant, and the sample sizes per habitat were small (N = 7 – 9 fish / population), we did not build more complicated models. For the same reason, we did not build a full model (because of the small sample size and high number of parameters, the residual degrees of freedom would be df=9, resulting in very low power).

Fig S5 Relationship between RA (dry ovary mass) and dry eviscerated mass in the four populations of *C. venusta*. One outlier is shown with filled circles; the regression lines were fitted by excluding the outlier

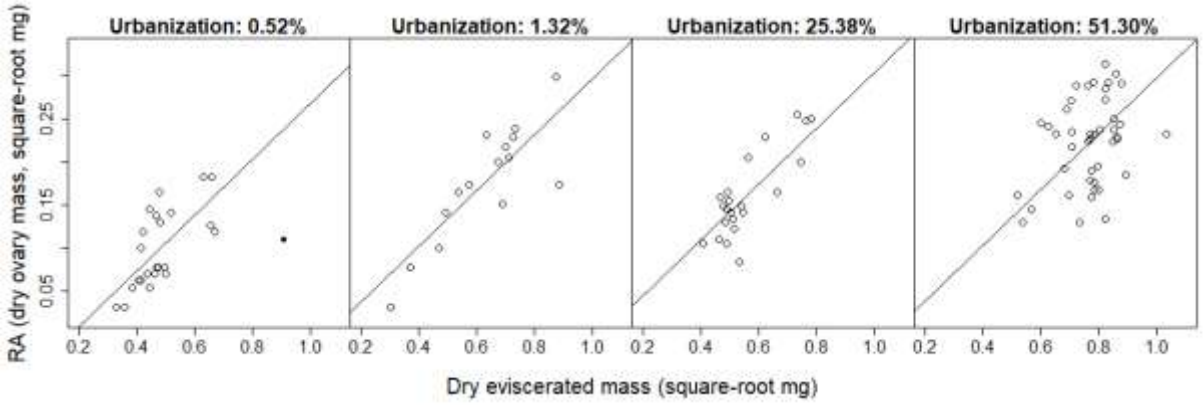

Table S5 GLS models of RA evaluated during forward model selection with type-2 analysis-of-deviance tables for *C. venusta*. The relevant term evaluated in each step is highlighted in bold

Model #	Model terms	df	χ^2	P
Model 1	Mass	1	96.67	<0.001
	Urbanization	3	14.36	0.002
	Mass × Urbanization	**3**	**5.62**	**0.131**
Model 2	Mass*	1	98.13	<0.001
	Urbanization	3	14.42	0.002
Model 3	Mass	1	66.69	<0.001
	Urbanization	2	0.27	0.872
	BaselineCort	**1**	**0.01**	**0.941**
Model 4	Mass	1	64.74	<0.001
	Urbanization	2	0.34	0.843
	StressResponse	**1**	**0.01**	**0.907**
Model 5	Mass	1	51.73	<0.001
	Urbanization	2	0.54	0.764
	BaselineCort	1	0.00	0.945
	StressResponse	1	0.14	0.712
	StressResponse × BaselineCort	**1**	**1.74**	**0.187**
Model 6	Mass	1	48.18	<0.001
	Urbanization	2	0.25	0.884
	BaselineCort	1	0.03	0.865
	Urbanization × BaselineCort	**2**	**2.87**	**0.238**
Model 7	Mass	1	62.97	<0.001
	Urbanization	2	0.53	0.768
	StressResponse	1	0.30	0.584
	Urbanization × StressResponse	**2**	**5.22**	**0.074**

The models exclude one outlier that violated the assumptions of linearity, normality and homoscedasticity (Fig. S5). "Mass" stands for dry eviscerated mass. Note that several variables were transformed (see Methods), and the model coefficients were not back-transformed to the original scale of the variables. Sample size was N = 103 in Models 1-2, and N = 24 in all other models.

*Slope (± SE) of the relationship between RA and mass: 0.358 ± 0.036 (t_{56} = 10.01)

Supplementary Results:

Table S6 Differences in cortisol release rates between consecutive treatments across all female *Gambusia affinis* and *Cyprinella venusta*, and within each site (labeled by the % of developed land), estimated from LMM models. Note that the differences and their standard errors are given on natural logarithmic scale

Species	Comparison	Site	Difference	SE	df	t	P
G. affinis	Baseline - Agitation	All fish	-0.509	0.039	215	-13.15	<0.001
		0.52%	-0.728	0.084	143	-8.65	<0.001
		1.32%	-0.107	0.088	143	-1.21	0.229
		5.24%	-0.612	0.087	143	-7.05	<0.001
		21.34%	-0.442	0.113	143	-3.92	<0.001
		25.38%	-0.541	0.064	143	-8.47	<0.001
		51.30%	-0.456	0.082	143	-5.59	<0.001
	Agitation - Recovery	All fish	0.041	0.053	215	0.77	0.445
		5.24%	-0.160	0.120	64	-1.34	0.186
		21.34%	0.045	0.117	64	0.38	0.703
		25.38%	0.112	0.107	64	1.05	0.298
		51.30%	0.294	0.145	64	2.03	0.047
C. venusta	Baseline - Agitation	All fish	-0.609	0.070	63	-8.77	<0.001
		0.52%	-0.543	0.209	60	-2.60	0.012
		1.32%	-0.924	0.110	60	-8.43	<0.001
		25.38%	-0.549	0.137	60	-4.01	<0.001
		51.30%	-0.432	0.112	60	-3.85	<0.001

Random effects:
G. affinis, variance among populations: 0.092, among individuals: 0.205, residual variance: 0.118
C. venusta, variance among populations: 0.205, among individuals: 0.425, residual variance: 0.145

Table S7 GLS model of the relationship between stress response and urbanization (% of developed land) in *G. affinis*, excluding the population that did not show significant stress response

Model coefficients	Estimate	SE	t	P
Intercept	0.470	0.131	3.60	0.001
Urbanization	-0.010	0.005	-2.06	0.042
Time	-0.006	0.007	-0.85	0.394

Sample size: N=129. 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. Date could not be included into this model because of high multi-collinearity (VIF>3) between date and urbanization in this subset of the data (in the presented model, VIF = 1.19)

Table S8 Pairwise differences between *G. affinis* sites (labeled by the % of developed land) for behavioral variables in 2019, estimated from GLS models

Dependent variable	Comparison	Difference	SE	df	t	P
Individual activity	5.24% - 21.34%	-0.810	0.583	37	-1.39	0.208
	5.24% - 25.38%	1.136	0.633	37	1.79	0.162
	5.24% - 51.30%	-0.735	0.496	37	-1.48	0.208
	21.34% - 25.38%	1.947	0.633	37	3.07	0.012
	21.34% - 51.30%	0.075	0.495	37	0.15	0.880
	25.38% - 51.30%	-1.872	0.553	37	-3.38	0.010
Group shoaling	5.24% - 21.34%	-0.162	0.410	18	-0.40	0.862
	5.24% - 25.38%	-1.596	0.291	18	-5.49	<0.001
	5.24% - 51.30%	-0.059	0.335	18	-0.18	0.862
	21.34% - 25.38%	-1.434	0.414	18	-3.46	0.006
	21.34% - 51.30%	0.103	0.447	18	0.23	0.862
	25.38% - 51.30%	1.537	0.341	18	4.50	0.001

Fig. S6 Behavior of *G. affinis* in individual and group tests in 2018 and 2019. Activity and shoaling were expressed as principal component scores. In each boxplot, the thick middle line and the box, respectively, show the median and interquartile range, and whiskers extend to the most extreme data points within 1.5 × interquartile range from the box

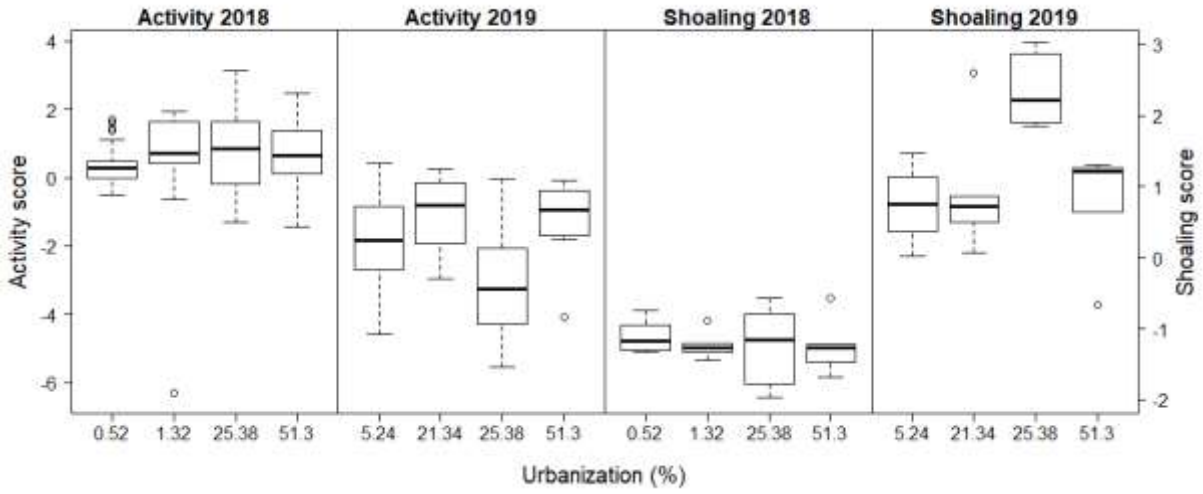

Table S9 Pairwise differences between *C. venusta* sites (labeled by the % of developed land) for glucocorticoid and life-history variables, estimated from GLS models

Dependent variable	Comparison	Difference	SE	df	t	P
Baseline cortisol release rate	0.52% - 1.32%	-0.630	0.242	56	-2.61	0.035
	0.52% - 25.38%	-0.245	0.246	56	-0.99	0.325
	0.52% - 51.30%	0.271	0.196	56	1.39	0.206
	1.32% - 25.38%	0.386	0.271	56	1.42	0.206
	1.32% - 51.30%	0.902	0.226	56	4.00	0.001
	25.38% - 51.30%	0.516	0.230	56	2.24	0.058
Stress response	0.52% - 1.32%	-5.921	4.000	56	-1.48	0.288
	0.52% - 25.38%	-0.371	4.200	56	-0.09	0.930
	0.52% - 51.30%	0.992	4.110	56	0.24	0.930
	1.32% - 25.38%	5.550	3.050	56	1.82	0.223
	1.32% - 51.30%	6.913	2.930	56	2.36	0.131*
	25.38% - 51.30%	1.362	3.200	56	0.43	0.930
RA	0.52% - 1.32%	-0.029	0.013	94	-2.28	0.066
	0.52% - 25.38%	-0.036	0.010	94	-3.77	0.002
	0.52% - 51.30%	-0.030	0.014	94	-2.17	0.066
	1.32% - 25.38%	-0.007	0.011	94	-0.61	0.764
	1.32% - 51.30%	-0.001	0.013	94	-0.11	0.916
	25.38% - 51.30%	0.006	0.012	94	0.47	0.764
Slope of relationship of RA with stress response	0.52% - 1.32%	0.002	0.002	14	1.39	0.282
	0.52% - 25.38%	0.004	0.002	14	2.24	0.126**
	1.32% - 25.38%	0.001	0.002	14	0.75	0.466

*Without FDR correction, this difference was significant at P = 0.022

**Without FDR correction, this difference was significant at P = 0.042

Table S10 The effect of gestational stage and percent development on baseline cortisol release rate across years.

Source	Nparm	DF	F Ratio	P
% Developed	1	1	43.476	<.0001
Gestation stage	1	1	0.0003	0.986
% Developed×gestation	1	1	1.138	0.288
Year	1	1	0.023	0.879

Fig. S7 The relationship between gestational stage and % development on baseline cortisol release rate across years.