

URBANIZATION AFFECTS THE PHYSIOLOGY, BEHAVIOR, AND
LIFE-HISTORY TRAITS OF A TOLERANT STREAM FISH

by

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ABSTRACT

Anthropogenic land-use conversion is one of the main drivers of wildlife population extinctions and biodiversity loss. Freshwater fishes are among the taxa most affected by land-use conversion, with an estimated 25% of species at risk of extinction. Urbanization is a rapidly growing form of land-use conversion that has been negatively impacting stream ecosystems. The change in stream quality associated with urbanization often pushes native and intolerant fish species past their physiological limits. As a result, urbanization alters fish distributions and assemblages by reducing the amount and diversity of intolerant species, and increasing the abundance and dominance of tolerant species. I explored the effects of urbanization on the physiology, life-history, and behavior of *Gambusia affinis*, a species of live-bearing fish that frequently inhabits urban streams. I also examined how context-specific variation in glucocorticoid physiology affected their reproductive allotment across urban environments. I collected *G. affinis* from four streams in 2018 and four streams in 2019, which differed in their degree of urbanization. There was twice as much rainfall in 2019 than 2018, which is known to exacerbate the effects of urbanization. I found that urban populations of *G. affinis* had more offspring and heavier broods than rural populations. Additionally, urban populations had higher baseline cortisol release rates, which explained differences in reproductive allotment in 2018 but not 2019. However, the interaction between physiological responsiveness and recovery explained differences in reproductive allotment in 2019. There were behavioral differences across populations and years. Fish

from the most urban site in 2018 spent less time moving compared to the three less urban populations and fish in 2019 differed in their shoaling behavior. My results suggest that baseline and stress-induced cortisol release rates play different roles in coping with environmental perturbations, depending on the severity and stochasticity associated with the environment. Lastly, I suggest that various components of glucocorticoid regulation help increase fitness across environments, thereby helping *G. affinis* to adapt and persist in urban streams.

I. LITERATURE REVIEW

The growing demand for resources associated with urban and agricultural development has resulted in a dramatic modification of Earth's natural landscapes. Anthropogenic land-use conversion disrupts the structure and functioning of ecosystems, and alters the way in which ecosystems interact (Vitousek et al., 1997). Coupled with land-use conversion, secondary processes such as environmental pollution and climate change can push ecosystems toward even further disequilibrium. Anthropogenic alterations to habitat through land-use conversion comprise one of the main drivers of wildlife population extinctions and biodiversity loss (Turner et al., 2007; Brook et al., 2008; Ceballos et al., 2015).

The freshwater biome, which includes 40% of Earth's biodiversity is especially sensitive to landscape modifications (Ricciardi & Rasmussen, 1999; Lundberg et al., 2000). Freshwater fauna are particularly harmed by human-mediated habitat destruction and are degrading at a rate five times faster than terrestrial fauna (Ricciardi & Rasmussen, 1999; Allan, 2004). Freshwater fishes are among the taxa most imperiled by the effects of land-use conversion, and it is estimated that worldwide 25% of freshwater fish are at risk of extinction (Miller et al., 1989; Ricciardi & Rasmussen, 1999; Vié et al., 2009). There is a growing need to better understand the effects of land use conversion on resident fishes in order to diminish future disturbances and prevent population declines.

Rivers and streams are strongly influenced by their surrounding landscapes (Hynes, 1975). Consequently, anthropogenic alterations to terrestrial habitat can have deleterious effects on freshwater systems that flow through modified landscapes (Allan, 2004). Human actions at the landscape scale disturb the biological integrity of freshwater

ecosystems, thereby impacting habitat, water quality, and biota through several complex pathways and interactions (Allan et al., 1997). The two main drivers of land use conversion are agriculture and urban development. Herein I will focus primarily on the effects of urbanization on freshwater rivers and streams.

The rapid growth of human civilization has resulted in a dramatic increase in the number of humans occupying urban areas and an increase in the number and size of urban areas themselves. According to a recent United Nations report on sustainable urban planning, two-thirds of the world's human population are expected to live in urban areas by the year 2050 (United Nations, 2014). While urban land use has traditionally encompassed a low percentage of total catchment area, metropolitan-area sprawl is impacting an increasing proportion of stream networks and exerting a disproportionately large impact on the stream ecosystems on multiple spatial scales (Paul & Meyer, 2001; Meyer et al., 2005). The continuous expansion of urban land cover results in more natural habitat loss and reduced biodiversity (While & Whitehead, 2103). Urbanization leads to an increase in the amount of impervious surface cover (e.g. driveways, roads, parking lots, rooftops, or any surface that cannot effectively absorb rainfall) within a watershed, which is the strongest predictor of stream water quality and ecological health (McMahon & Cuffney, 2000; Wang et al., 2001). The ecological effects brought about by streams draining urban catchments are collectively known as “the urban stream syndrome” (Walsh et al., 2005). The symptoms of the urban stream syndrome include altered hydrology, elevated concentrations of nutrients and contaminants, altered channel morphology, reduced biotic richness, and the dominance/presence of more tolerant species (Paul & Meyer, 2001; Meyer et al., 2005; Walsh et al., 2005).

An increase in the amount of impervious surface cover within a watershed leads to a decrease in infiltration and an increase in surface runoff (Dunne & Leopold, 1978). In fact, Arnold and Gibbons (1996) found that a 10-20% increase in impervious surface cover results in a twofold increase in surface runoff, a 35-50% increase in impervious surface cover results in a threefold increase in surface runoff, and a 75-100% increase in impervious surface cover results in a fivefold increase in surface runoff. Increases in impervious surface cover and surface runoff lead to consistent hydrology changes. Specifically, streams develop more frequent, larger and faster flow events which are driven primarily by stormwater drainage systems (Walsh et al., 2005). In Texas streams, Espey et al. (1966) found that an increase in impervious surface cover leads to an increase in peak discharge and a decrease in lag time, directly contributing to flood magnitude. The increased frequency and magnitude of stream flows are known to negatively impact aquatic biota, particularly fish assemblages (Booth, 2005; Roy et al., 2005). Increased flood magnitude can directly impact fish assemblages by washing away eggs, larvae, and young, thereby disrupting their life-cycles (Roy et al., 2005). Altered hydrology also contributes to altered channel dynamics via bank erosion and sediment deposition, thereby directly degrading natural fish spawning and foraging habitats (Paul & Meyer, 2001; Allan, 2004). Moreover, warming of surface runoff along with riparian clearing alters stream thermal regimes, which can be unfavorable as fish are ectothermic and thermal refugia are an important resource for maintaining physiological, metabolic, and behavioral homeostasis (Magnuson et al., 1979; Huey, 1991; Allan, 2004).

Urbanization also affects stream water quality. Specifically, urban streams exhibit a significant increase in conductivity, total dissolved solids, oxygen demand, and

pollutants primarily associated with waste water treatment plant effluent and non-point source runoff (Paul & Meyer, 2001). The EPA's 2000 National Water Quality Inventory Report identified pollutants associated with urban runoff as one of the leading sources of water-quality impairment in surface waters (U.S. Environmental Protection Agency, 2002). The range of pollutants entering urban streams is variable, but include road salts, poly-aromatic hydrocarbons, pesticides, sediment, heavy metals, viruses, and bacteria (Richards et al., 2010). Urban streams also exhibit a large amount of nutrient loading, particularly in the form of nitrogen and phosphorus (Paul & Meyer, 2001). Reduced water quality and pollutant exposure can impact fish by altering endocrine signaling, stress physiology, behavior, life-history traits, and olfaction (Weis et al., 1999; Edwards & Guillette, 2007; Tierney et al., 2010; Blevins et al., 2013; Jeffrey et al., 2015).

Urbanization is associated with significant shifts in fish health and community structure (Paul & Meyer, 2001; Allan, 2004; Walsh et al., 2005). Fishes in urban streams tend to have a greater amount of eroded fins, lesions, tumors, and physiological markers of stress with increasing urbanization (Helms et al., 2005; Jeffrey et al., 2015; King et al., 2016). The change in stream quality associated with urbanization exposes fish, particularly native and sensitive species, to stressful conditions that often push them past their physiological limits (Adams et al., 2002). As a result, urbanization alters fish distributions and assemblages by reducing the amount and diversity of sensitive species, and increasing the abundance and dominance of tolerant species (Karr, 1981; Wang et al., 2003; Walsh et al., 2005). The extirpation of sensitive fish species is a commonality in urban streams (Paul & Meyer, 2001). Furthermore, a growing number of researchers have commented on the physiological, morphological, and behavioral differences between

individuals found in rural and urban areas (Casten & Johnston, 2008; South & Ensign, 2013; French et al., 2018; Santangelo et al., 2018; Kern & Langerhans, 2019; Ouyang et al., 2019). Understanding how fish populations respond to urbanization and cope with increasing environmental perturbations has important evolutionary and ecological implications.

Physiological response to stress

Urbanization can affect the stress physiology of aquatic organisms, primarily through reducing the quality of habitat, decreasing connectivity, and limiting essential resources (Jeffrey et al., 2015). One widely proposed mechanism that explains why certain individuals can persist in urban habitats involves the vertebrate endocrine system (Ouyang et al., 2019). In particular, glucocorticoid (GC) hormones mediate an organism's response to both predictable and unpredictable changes in the environment (Romero et al., 2009; Guindre-Parker, 2018), thereby facilitating adaptive physiological, behavioral, and morphological responses to environmental perturbations (Wingfield & Kitaysky, 2002). Moreover, as GCs mediate both internal metabolic regulation and adaptive responses to external challenges, they can have a profound effect on an organism's fitness (Shoenle et al., 2018). However, while variation in the endocrine response to environmental perturbations has often predicted the underlying differences among individuals and species in their capacity to cope with stressors, determining precisely when and how GC regulation influences fitness remains unclear (Vitousek et al., 2019). There can be positive, negative, and neutral relationships between GCs and fitness, which can vary within a single species among years, life-history stages, sex, and resource availability (Breuner et al., 2008; Bonier et al., 2009; Shoenle et al., 2018;

Vitousek et al., 2018). Nevertheless, analyzing physiological stress response in fish can be a useful method to evaluate stream quality and the consequences of urbanization (Barton, 2002; Jeffrey et al., 2015; King et al., 2016).

The stress response is an important and energetically costly hormone-mediated mechanism that allows organisms to maintain homeostasis and adjust their behavioral, physiological, and life-history phenotypes in response to environmental disturbances (Romero, 2004). Fish evoke a glucocorticoid stress response that facilitates mobilizing energy stores, and mediates rapid behavioral and physiological changes when coping with acute environmental perturbations (Wendelaar Bonga, 1997; Barton, 2002). In response to acute stressors, cortisol (the primary GC in fish) is transiently elevated, which helps maintain homeostasis by temporarily increasing energy metabolism, fueling muscles with oxygen and glucose, preventing tissue damage, and moderating immune and reproductive functionality (Wendelaar Bonga, 1997; Barton, 2002; Romero, 2004). Further, elevated cortisol aids fishes by increasing gill surface area, thereby maximizing oxygen uptake during low oxygen conditions and balancing ion concentrations through osmoregulation (McDonald et al., 1991).

Glucocorticoids are crucial mediators of individual phenotypic flexibility and are important for coordinating adjustments to environmental variations (Nelson, 2011; Hau et al., 2016). However, while acute GC release can be beneficial, exposure to prolonged or chronic stressors can become maladaptive and lead to long-term suppression of growth, reproduction, and immune function (Sapolsky et al., 2000). Chronic stress and/or exposure to pollutants can also result in a muted stress response, or failure of an organism

to respond to subsequent stressors which becomes maladaptive to survival (Barton, 2002; Romero, 2004).

The stress response begins with an integration of stimuli in the brain, leading to a secretion of corticotropin-releasing factor (CRH) by the hypothalamus. CRH regulates the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland, which then stimulates the release of GCs from the interrenal cells (Wingfield & Romero, 2001). The GCs circulate throughout the bloodstream until they bind to glucocorticoid and mineralocorticoid receptors within their target tissues and elicit the appropriate physiological or behavioral response (Wendelaar Bonga, 1997; Sapolsky et al., 2000; Barton, 2002). The end of the stress response is regulated through the inactivation of GC release via a negative feedback loop when GCs bind to their receptors in the hypothalamus, hippocampus, or pituitary gland (Nelson, 2011). The physiological response of fish to environmental stressors, as reviewed by Barton (2002), can be grouped into three levels: primary, secondary, and tertiary stress response. The primary stress response involves the release of GC and catecholamine hormones into circulation. Direct stressors and circulating hormones activate the secondary stress response, thereby altering the fish's metabolic, cellular, osmoregulatory, hematological, and immune profiles. The secondary stress response involves increasing plasma glucose and lactate, decreasing tissue glycogen, increasing production of heat-shock proteins, altering ion balance, changing blood-cell profiles, and disrupting lysozyme activity and antibody production. Lastly, the tertiary stress response affects whole-organism fitness by modulating growth, body condition, disease resistance, locomotion, foraging ability, reproduction, behavior, and ultimately survival (Barton, 2002).

The regulation of the GC stress response will herein be called the hypothalamic-pituitary-interrenal (HPI) axis. Short term release and inhibition of GCs for internal maintenance or in response to acute stressors is necessary for maintaining homeostasis. This healthy process of regulation is known as reactive homeostasis (Romero et al., 2009). However, severe or prolonged stressors can cross a pathological threshold and dysregulate the HPI axis, causing permanently elevated or depressed levels of GCs. The animal can then enter a state of homeostatic overload, which results in the inability to mount a stress response to novel stressors, problems with metabolic, reproductive, and immune function, and ultimately death (Romero et al., 2009). One non-invasive method for assessing the responsiveness of the HPI axis is to illicit a GC response to a novel stressor by acutely agitating individuals over a short period of time (Gabor et al., 2013). If an individual does not show a stress response to agitation, then they are likely experiencing chronic stress as indicated by dysregulation of the HPI axis. However, some species of fish are more resilient than others and may even habituate to repeated stressors, thereby continuing to maintain populations even in poor quality habitats (Barton, 2002; Cyr & Romero, 2009; King et al., 2016; Wingfield, 2013). Assessing the fish stress response across multiple species and habitat quality gradients can give scientist and land managers a better understanding of stream ecosystem health and dynamics in response to urbanization (Barton, 2002; Jeffrey et al., 2015; Blevins et al, 2013; King et al., 2016).

How stressors influence circulating levels of GCs depends on the responsiveness or dysregulation of the HPI axis, and the ability of an organism to modulate its response to a perturbation (Cyr & Romero, 2009). Unless environmental stressors are severe enough to permanently alter or challenge a fish's homeostatic mechanisms beyond their

limits, physiological processes can generally adapt to compensate for stress (Barton, 2002). The stress response can evolve as part of a behavior and life-history strategy (Crespi et al., 2013), and there can be selection for phenotypes capable of buffering extreme conditions while still maintaining reproductive output (Martin & Wiebe, 2004; Crespi et al., 2013; Wingfield, 2013). These adaptations are known as the perturbation resistance potential, or the ability of individuals to experience disturbances yet continue to maintain normal life processes (Wingfield et al., 2011; Wingfield, 2013). Wingfield (2013) outlines the three major components of the perturbation resistance potential, which can be regulated and can vary among individuals, populations, and life-history stages: modulation of robustness, modulation of responsiveness, and modulation of resilience. Modulation of robustness is the ability of an organism to become more resistant to acute disturbances, and thus avoiding triggering behavioral and physiological responses (Wingfield, 2013). Moreover, repeated exposure to the same disturbance can result in habituation of the individual to the stressor (Cyr & Romero, 2009). Habituated animals can learn to perceive the repeated stressor as innocuous and therefore reduce the intensity of their stress response to that particular stimulus (Cyr & Romero, 2009). Modulation of responsiveness is the ability of an organism to more flexibly regulate its actual response to stress through adjustments of behavioral and physiological responses (Wingfield, 2013). Modulation of resilience refers to how rapidly and efficiently an organism can recover back to baseline GC levels after a perturbation has passed (Wingfield, 2013). In fluctuating environments, individuals that can continuously but reversibly alter their behavior, physiology, and life-history, will have increased fitness and incur a selective advantage over individuals that are less flexible (Piersma & Drent,

2003). However, if an organism cannot acclimate to disturbances, then reduced fitness or death may occur. This paradigm supports the reasoning behind why more tolerant generalist fish species dominate fluctuating lower quality urbanized environments. It can be useful to examine modulation of the stress response to better understand how organisms can cope with or recover from stressors, including those imposed by urbanization.

Physiology and life-history

Variations in environmental quality can influence the phenotypes and performance of organisms, which can impact life-history traits and provide a link between habitat and fitness (Ricklefs & Wikelski, 2002). The life-history traits of fish, particularly those related to reproduction, can vary across environmental gradients (Heins, 1991; Mazzoni & Iglesias-Rios, 2002), and exposure to stressors associated with urbanization can negatively affect the reproductive traits in fish (Johnston & Farmer, 2004; Cazan & Klerks, 2015). Not only does urbanization destroy spawning habitat for egg-laying species (Allan, 2004), but dissolved pollutants from waste water treatment plant effluent and runoff are easily absorbed, causing damage to somatic and reproductive tissues (Dutta & Meijer, 2003; Edwards & Guillette, 2007; Kidd et al., 2007; Hostovsky et al., 2014). Furthermore, elevated baseline GC levels among individuals in the same life-history stage are often correlated with poor body condition and lower reproductive output (Romero, 2002). Elevated cortisol has been shown to inhibit vitellogenesis in female rainbow trout (*Oncorhynchus mykiss*) and suppress reproductive hormones in multiple fish species (Lethimonier et al., 2000; Barton, 2002).

However, stressors can affect a fish's life-history traits in various ways, largely depending on the severity and duration of the stressor, and when in the life cycle the stressor is encountered (Schreck, 2010). A fish's ability to respond to stressors develops very early in life (Barton, 2002), and can have a strong effect on their phenotypes that last into adulthood (Sheriff & Love, 2013). It is not always clear whether fishes that display high GC concentrations are more stressed than others, or whether they have different capacities to respond to stress (Barton, 2002). Elevated GC levels do not always interfere with reproductive investment, and in many situations may actually facilitate it. Differing life-history strategies may select for stress resistance in environments that are consistently adverse (Crespi et al., 2013). Adjustments to life-history strategies in response to urbanization and stress may also differ across individuals and species (Heins, 1991; Casten & Johnston, 2008). Depending on the situation, perceived stress could either inhibit reproduction or accelerate ovulation (Schreck, 2010). As a result, more tolerant species of fish may modulate their life-history traits and continue to persist in urbanized environments (South & Ensign, 2013; Casten & Johnston, 2008) while more sensitive species perish.

Several investigators have incorporated life-history theory into predicting how certain fishes respond to environmental change (Stearns 1983; Winemiller & Rose, 1992; Van Winkle et al., 1993). The life-history of an organism is an integrated allocation of energy gained from food, to the expenditure of energy lost through maintenance, activity, growth, and reproduction (Vondracek et al., 1988). Life-history theory predicts that the energy allocated to these competing needs should maximize fitness during an organism's lifetime (Giesel, 1976). Furthermore, life history evolution suggests that different life-

history components are causally related, so that an increase in a particular life-history trait can be associated with a decrease in another (Reznick, 1983). Variation in environmental factors can affect the allocation of energy to certain life-history traits, illuminating the adaptive nature of these plastic traits in different situations (Giesel, 1976; Vondracek et al., 1988). Studies of life-history evolution typically focus on phenotypic variation among populations or among species (Johnson and Bagley, 2011). In fish populations, trade-offs in life-history traits are evolutionary responses to different kinds of environments and disturbances (Balon, 1975; Winemiller & Rose, 1992). A common example of this trade-off is an increase in reproductive effort resulting in decreased growth and survivorship (Stearns, 1989). Reznick & Endler (1982) reported that guppies (*Poecilia reticulata*) allocated more resources towards reproduction, had a shorter brood interval, matured earlier, and birthed smaller young when exposed to high predation environments. Stearns (1983) reported that *Gambusia affinis* living in streams with higher water fluctuations matured earlier, produced more eggs and had a higher reproductive investment than their counterparts that were raised in stable environments. The observed differences in life-history phenotypes among populations result from heritable variation, phenotypic plasticity, or a combination of the two (Johnson & Bagley, 2011). Most of these life-history trait fluctuations are mediated by hormones, yet their mechanisms require further exploration (Stearns, 1989).

Physiology and behavior

Variation in circulating levels of GCs along with other hormonal and behavioral responses to changing environments have been observed both within and across species (Hau et al., 2016). Glucocorticoids elicit behavioral responses that are mediated by

genomic and non-genomic effects in the brain (Øverli et al., 2001). Sustained exposure to elevated cortisol levels may contribute to suppressed feeding and locomotor activity in fish (Gregory & Wood, 1998) and amphibians (Crespi & Denver, 2005). Elevated GC levels during a stress response and the decreased capacity to recover from an acute stress response had been associated behavioral alterations including decreased locomotion, impairment in learning, and a general state of fearfulness (Hau et al., 2016). Moreover, Endocrine disrupting compounds (EDCs) that enter urban streams via runoff can interfere with the endocrinology and behavior of freshwater fish (Edwards & Guillette, 2007; Knapp et al., 2011; Saaristo et al., 2014). EDCs are known to alter sexual behavior, which can impose important ecological and evolutionary consequences (Bertram et al., 2015; Saaristo et al., 2014; Toft et al., 2003). Studies conducted by Bertram et al. (2015) and Saaristo et al. (2014) show altered male reproductive behavior in two different species of viviparous fish as a result of ecologically relevant EDC contamination. In both studies, male fish from land converted environments exhibited increased courtship and forced copulatory behavior towards females.

Metabolic rate, defined as the rate at which an animal oxidizes substrates to produce energy and maintain physiological homeostasis is a fundamental measure in ecology and evolution (Brown et al., 2004). Comparative studies conducted by McNab (2002) and Lovegrove (2003) show that metabolic rate is an ecologically important physiological trait that can evolve as species respond to different ecological conditions. Differences in metabolic rate between individuals may be related to differences in behavior such as boldness, aggressiveness, reactivity, sociability, exploration or activity levels (Réale et al., 2007). In *Gambusia affinis*, bolder, more active individuals explore

more, are more social (school more), and disperse further (Cote et al. 2010). Individuals that are more explorative, aggressive, and bold may expend energy at higher rates than individuals that do not display such behaviors (Lovegrove, 2003). Furthermore, higher metabolic rates allow more active and bold individuals to sustain greater muscular activity and cellular machinery (Careau et al., 2008). These individuals tend to be more successful in competing for resources and therefore grow faster, and reproduce earlier (Cote et al., 2010; Polverino et al., 2018). Studying differences in fish behavior between natural and disturbed habitats may be informative because of the underlying physiological correlates and likely relation to fitness (Koolhaas et al., 1999; Dingemanse & Reale, 2005; Polverino et al., 2018).

Conservation physiology

Understanding the relationship between GCs and fitness can give researchers better insights on how animals cope with stressors and what constitutes a successful physiological stress response (Breuner et al., 2008; Shoenle et al., 2018). If GCs are associated with environmental challenges and fitness, then they can function as indicators of stress and individual/population health (Dantzer et al., 2014). Moreover, GCs can be easily measured in many species, which makes them a valuable tool for monitoring the health of wildlife populations and aiding in conservation efforts (Dantzer et al., 2014; Shoenle et al., 2018). However, although the overall GC response is highly conserved in vertebrates, the response of the HPI axis to challenges can vary among individuals, populations, and species (Shoenle et al., 2018). The equivocal nature of the HPI axis is documented in the variability of circulating baseline and stress-induced GCs, as well as the efficacy of negative feedback across populations, species, and contexts (Bonier et al.,

2009; Romero & Wikelski, 2010; Cockrem, 2013; Guindre-Parker, 2018). Documenting the phenotypic plasticity in the GC stress response, life-history traits, and behavior is the first step towards understanding the persistence or extirpation of fishes exposed to various environmental conditions.

Natural history of *Gambusia affinis*

Gambusia affinis, commonly known as the Western Mosquitofish, are small viviparous freshwater fish in the family Poeciliidae. Having been introduced from North America, *G. affinis* are now present and widely distributed across every continent except for Antarctica (Lloyd, 1986). They are extremely hardy and flexible regarding habitat preference, but are generally restricted to low elevations with warmer waters (Pyke, 2008). In the United States they range in the southern half of the country, east of the Rocky Mountains and west of the Appalachian Mountains. They are found throughout the state of Texas (Hubbs et al., 1991).

Males and females are sexually dimorphic. Females typically reach a maximum size of 5-6 cm in standard length (SL) (Hughes, 1985a), whereas males typically reach a maximum size of 3 cm (Hughes, 1985b). Females can live up to 1.5 years in the wild, whereas the lifespan of males is much shorter (Daniels & Felley, 1992; Haynes & Cashner, 1995). Males have an external organ called a gonopodium; a modified anal fin that is used to passage sperm to a females urogenital opening during mating (Meffe & Snelson, 1989).

During copulation, a male will approach the female from the side or from behind and thrust his gonopodium forward, transferring sperm by momentarily inserting the tip into the urogenital opening of the female (Peden, 1975). After copulation, females can

store sperm for a lifetime, adjusting the timing of fertilization, and multiple broods can be produced from a single mating (Haynes, 1993). Once the eggs are fertilized, young are typically born 21-28 days after 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 & Cashner, 1995). Females are also capable of breeding for a second season (Maglio & Rosen, 1969). Males are capable of successfully mating at any time throughout the breeding season (Fraile et al., 1992). Giving birth to live young may result in a higher survival rate as compared to egg-laying fish, and population size can therefore increase very rapidly (Pyke, 2008).

Several investigators have found significant differences in size and number of offspring of female *Gambusia affinis*, which they attributed to both genetic differences and plastic responses to varying environments (e.g., Stearns, 1983; Reznick et al., 1990). *Gambusia affinis* are known to thrive in a wide range of habitats across a broad range of landscapes and environments, often ones that exhibit physical, chemical, and biological characteristics that are generally unfavorable to most freshwater fish species (Lloyd, 1986; Hubbs, 2000; Pyke, 2005). Cherry et al. (1976) noted that *G. affinis* are able to tolerate water temperatures from 0 to 45° C, dissolved oxygen concentrations from approximately 1 to 11 mg/l, and turbidity from approximately 3 to 275 Jackson Turbidity Units. Hubbs (2000) demonstrated that *G. affinis* are able to survive at least a week in salinities over 41 ppt, total ammonia over 10 ppm, and dissolved oxygen below 1 ppm, all of which can be lethal to many endemic fish species. Additionally, *G. affinis* are able to tolerate pH ranges from approximately 4.5 to 9 (Keup & Bayliss, 1964). They occur in

water bodies that range from undisturbed streams, lakes, and swamps to highly disturbed environments including heavily polluted and overgrown urban drainage systems (Cherry et al., 1976; Pyke, 2008). *Gambusia affinis* often exhibit increased abundance in disturbed habitats close to or within urban areas, which may have also marked them as a favorable candidate for mosquito control (Moore, 1922; Pyke, 2008). They are more resistant to toxic chemicals and pollutants associated with urbanized environments, as compared to other fish species (Lloyd et al., 1986). Although *G. affinis* are tolerant to a wide range of habitats, they typically prefer relatively warm, low elevation water bodies and do not typically colonize estuaries. They are absent from ephemeral aquatic environments, and their densities and occurrences may be limited by the presence of large predatory fish (Pyke, 2008). Not only can *G. affinis* colonize many different habitats, but they often do so in great abundance (Morton et al., 1988). However, while widely occurring, *G. affinis* are not equally abundant across all habitats and temporal scales. For example, Hahn (2017) compiled records and recent abundances of *G. affinis* within the Chihuahuan Desert, Edwards Plateau, and South Texas Plains ecoregions of Texas, and found that their abundances ranged widely from rare to common. The abundance and adaptive capabilities of the mosquitofish are governed by their general biology, phenotypic plasticity, and considerable variability within and among populations on a spatial and temporal scale.

II. URBANIZATION AFFECTS THE PHYSIOLOGY, BEHAVIOR, AND LIFE-HISTORY TRAITS OF A TOLERANT STREAM FISH

Introduction

The growing demand for resources associated with urban and agricultural development has resulted in a dramatic modification of Earth's natural landscapes. Anthropogenic alterations to habitat through land-use conversion comprise one of the main drivers of wildlife population extinctions and biodiversity loss (Turner et al., 2007; Brook et al., 2008; Ceballos et al., 2015). The freshwater biome, which includes 40% of Earth's biodiversity is especially sensitive to landscape modifications (Ricciardi & Rasmussen, 1999; Lundberg et al., 2000). Freshwater fishes are among the taxa most imperiled by the effects of land-use conversion, and it is estimated that worldwide 25% of freshwater fish are at risk of extinction (Miller et al., 1989; Ricciardi & Rasmussen, 1999; Vié et al., 2009).

Urbanization in particular is a rapidly growing form of land-use conversion that has led to the destruction of natural habitat and reduced biodiversity (While & Whitehead, 2103). While urban land use has traditionally encompassed a low percentage of total catchment area, metropolitan-area sprawl is impacting an increasing proportion of stream networks and exerting a disproportionately large impact on the stream ecosystems on multiple spatial scales (Paul & Meyer, 2001; Meyer et al., 2005). The ecological effects brought about by streams draining urban catchments are collectively known as "the urban stream syndrome" (Walsh et al., 2005). The symptoms of the urban stream syndrome include altered hydrology, elevated concentrations of nutrients and contaminants, altered channel morphology, reduced biotic richness, and the

dominance/presence of more tolerant species (Paul & Meyer, 2001; Meyer et al., 2005; Walsh et al., 2005). Many studies report that the effects of the urban stream syndrome are manifested when urban land cover reaches 10-20% (imperviousness) of the catchment area (Klein, 1979; Booth & Jackson, 1997; Paul & Meyer, 2001).

The change in stream quality associated with urbanization exposes fish, particularly native and intolerant species, to stressful conditions that may push them beyond their physiological limits (Adams et al., 2002). As a result, urbanization alters fish distributions and assemblages by reducing the amount and diversity of intolerant species, and increasing the abundance and dominance of tolerant species (Karr, 1981; Wang & Lyons, 2003; Walsh et al., 2005). The tolerance of a fish has historically been classified based on expert opinion, with tolerant species being those that are not sensitive to organic enrichment and low dissolved oxygen concentrations associated with water quality degradation (Linam et al., 2002). Further, studies have found physiological, morphological, and behavioral differences between conspecific individuals found in rural versus urban areas (Casten & Johnston, 2008; South & Ensign, 2013; King et al., 2016; Gabor et al., 2018; Santangelo et al., 2018; French et al., 2018; Kern & Langerhans, 2019; Ouyang et al., 2019). Understanding how fish populations respond to urbanization and cope with increasing environmental perturbations can provide a better understanding of urban evolution and inform decisions for sustainable land development and conservation.

One widely proposed mechanism that explains why certain individuals can persist in urban habitats involves the vertebrate endocrine system (Ouyang et al., 2019). In particular, glucocorticoid (GC) hormones produced by the hypothalamic-pituitary-

interrenal (HPI) axis mediate the response of an organism to both predictable and unpredictable changes in the environment (Romero et al., 2009; Guindre-Parker, 2018), thereby facilitating adaptive physiological, behavioral, and morphological responses to environmental perturbations (Wingfield & Kitaysky, 2002). As GCs mediate both internal metabolic regulation and adaptive responses to external challenges, they can have a profound effect on an organism's fitness (Shoenle et al., 2018). Yet while variation in the endocrine response to environmental perturbations has often predicted the underlying differences among individuals and species in their capacity to cope with stressors, determining precisely when and how GC regulation influences fitness remains unclear (Vitousek et al., 2019). There can be positive, negative, and neutral relationships between GCs and fitness, which can vary within a single species among years, life-history stages, sex, and environmental quality (Breuner et al., 2008; Bonier et al., 2009; Hau et al., 2016; Shoenle et al., 2018; Vitousek et al., 2018). Nevertheless, exploring GC regulation and its relationship to fitness may aid in understanding the costs and benefits of the GC response and provide insights into the capacity to cope with perturbations, including those imposed by urbanization.

Glucocorticoids are crucial mediators of individual phenotypic flexibility and are important for coordinating adjustments to environmental variations (Nelson, 2011; Hau et al., 2016). In response to acute stressors, cortisol (the primary GC in fish) is transiently elevated, which helps maintain homeostasis by temporarily increasing energy metabolism, fueling muscles with oxygen and glucose, preventing tissue damage, and moderating immune and reproductive functionality (Wendelaar Bonga, 1997; Barton, 2002; Romero, 2004). Furthermore, elevated cortisol aids fishes by increasing gill surface

area, thereby maximizing oxygen uptake during low oxygen conditions and balancing ion concentrations through osmoregulation (McDonald et al., 1991). 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).

While acute GC release can be beneficial, exposure to prolonged or chronic stressors can lead to a pathological state of long term GC elevation, resulting in the suppression of growth, reproduction, and immune function (Sapolsky et al., 2000), and hinder behavioral and physiological processes that impact fitness (Wingfield & Sapolsky, 2003). Therefore, effectively coping with stressors should involve a balance between mounting a robust GC response (responsiveness) and effectively terminating the response (recovery) to return to normal behaviors and physiological processes (Wingfield, 2013; Vitousek et al., 2019; Fig. 1a). However, it is not exactly clear how populations cope when facing frequent or prolonged disturbances (high perturbations). An organism's capacity to cope could confer a fitness benefit when they have a stronger recovery (Wingfield, 2013), especially if the population is facing frequent or chronic disturbances that do not directly threaten their survival (Vitousek et al., 2019). Yet it is also possible that weaker recovery will increase the sensitivity of an organism to environmental cues and likelihood of reproductive success (Lattin et al., 2016), especially in populations with more flexible reproductive strategies (Vitousek et al., 2019). Additionally, exposure to high perturbation environments and/or pollutants can result in a muted stress response, or failure of an organism to respond to subsequent stressors which becomes maladaptive to survival and fitness (Barton, 2002; Romero, 2004). Therefore, it is important to explore

the possible relationships and interactions between responsiveness and recovery with fitness (Fig.1b). A positive relationship between responsiveness and recovery with fitness in low perturbation environments may confer a benefit to fitness as GCs rapidly increase and rapidly subside following a disturbance. There may be a trade-off in moderately perturbed environments whereby individuals show a positive relationship between fitness and responsiveness, but a cost to fitness in terms of the rate of recovery from a stressor, or vice versa. In high perturbation environments there may be negative relationships between both responsiveness and recovery with fitness. Being able to differentiate between and explore these different levels of coping would aid in understanding why some species and populations persist in an environment while others do not.

Variations in environmental quality can influence the phenotypes and performance of organisms, which can impact life-history traits and provide a link between habitat and fitness (Ricklefs & Wikelski, 2002). Several investigators have incorporated life-history theory into predicting how certain fishes respond to environmental change (Stearns 1983; Winemiller & Rose, 1992; Van Winkle et al., 1993). The life-history of an organism is an integrated allocation of energy gained from food, to the expenditure of energy lost through maintenance, activity, growth, and reproduction (Vondracek et al., 1988). Life-history theory predicts that the energy allocated to these competing needs should maximize fitness during an organism's lifetime (Giesel, 1976). Furthermore, life-history evolution suggests that different life-history components are causally related, so that an increase in a particular life-history trait can be associated with a decrease in another (Reznick, 1983). Variation in environmental factors can affect the allocation of energy to certain life-history traits,

illuminating the adaptive nature of these plastic traits in different situations (Giesel, 1976; Vondracek et al., 1988). In fish populations, trade-offs in life-history traits are evolutionary responses to different kinds of environments and disturbances (Balon, 1975; Winemiller & Rose, 1992). While most of these life-history trait fluctuations are mediated by hormones, their mechanisms require further exploration (Stearns, 1989).

Variation in circulating GCs along with other hormonal and behavioral responses to changing environments have been observed both within and across species (Hau et al., 2016). Elevated GC levels during a stress response and the decreased capacity to recover from an acute stress response have been associated with behavioral alterations including decreased locomotion, impairment in learning, and a general state of fearfulness (Hau et al., 2016). Moreover, endocrine disrupting compounds (EDCs) that enter urban streams via runoff can interfere with the endocrine signaling and affect the behavior of freshwater fish (Edwards & Guillette, 2007; Knapp et al., 2011; Saaristo et al., 2014). Individual variation in behavioral traits such as aggressiveness, sociability, exploration, activity, risk-taking, and exploring novel environments among conspecifics can affect many aspects of ecology (Sih et al., 2012). In *Gambusia affinis*, individuals that are more active and take more risks tend to explore more, are more social (shoal more), and disperse further, which may contribute to their invasiveness (Cote et al., 2010). These individuals also tend to be more successful in competing for resources and therefore grow faster, and reproduce earlier (Cote et al., 2010; Polderino et al., 2018). Studying differences in fish behavior between natural and disturbed habitats may be informative because of the underlying physiological correlates and likely relation to fitness (Koolhaas et al., 1999, Dingemanse & Réale, 2005; Polderino et al., 2018).

The purpose of our study was to assess the effects of urbanization on the behavior, physiology, and life-history of the Western Mosquitofish (*Gambusia affinis*), a widespread and globally invasive species of viviparous freshwater fish that often have greater abundance in disturbed habitats close to or within urban areas (Moore, 1922; Pyke, 2008). *Gambusia affinis* thrive in a wide range of habitats across a broad range of landscapes and environments, often ones that exhibit physical, chemical, and biological characteristics that are generally unfavorable to most freshwater fish species (Lloyd, 1986; Hubbs, 2000; Pyke, 2005). Although this species has been characterized as being tolerant based on expert opinion (Linam et al., 2002), the mechanisms on how and why *G. affinis* and other tolerant species respond favorably to poor quality environments is unknown. There is also a gap in knowledge regarding the effects of urbanization on viviparous fish. Quantifying the regulation of the HPI axis in *G. affinis* as it relates to fitness across a gradient of urbanization will aid in understanding the tolerance of this species and give insight on their capacity to cope with environmental change.

We explored the relationships between GCs, life-history, and behavior by sampling six populations of *G. affinis* across two years along a gradient of urbanization, as defined by percent development (imperviousness). We predicted that more urban populations of *G. affinis* would have a life-history trade-off for more eggs and embryos, yet of a smaller size, compared to their rural counterparts. Additionally, more urban populations would be more active, and more social, and exhibit more exploratory behavior in novel environments than their rural counterparts. Urban populations may support this increased energetic regulation by elevating their baseline cortisol levels. Because this species is considered to be a tolerant habitat generalist, we predicted each

population to demonstrate a healthy acute cortisol response to stress, indicating that they are not chronically stressed by their environment. We predicted that populations of *G. affinis* residing in less urban environments would have a greater capacity to cope and positive relationships between responsiveness and recovery with fitness, while populations in more urban environments would show lower capacity to cope and negative relationships between responsiveness and recovery with fitness. Documenting the phenotypic flexibility in the GC response to stress, life-history traits, and behavior is the first step towards understanding the persistence or extirpation of fishes exposed to various environmental conditions.

Materials and methods

We collected fish from six streams located within the Edward's Plateau region of Central Texas (Fig. 2; Table 1). We collected *G. affinis* from two more urbanized streams and two less urbanized streams from 22 May to 11 June, 2018. In 2019, we collected *G. affinis* from four streams that also varied in their degree of urbanization. There was much more rainfall during the Spring of 2019 compared to the Spring of 2018 (average of 49.9 cm compared to 27.7 cm from March – June; US Climate Data; Austin, TX), so we could not sample until 22 June to 2 July, 2019. Two of the streams that we sampled 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 that were previously sampled in 2018. We partially chose sampling sites based on a visual assessment of the percent impervious surface cover within the subwatershed surrounding each stream, 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 a watershed imperviousness of 10 – 20% (Paul & Meyer, 2001).

We collected female *G. affinis* (2018: n = 20; 2019: n = 18) using dipnets and seines at each site for water-borne hormone measurements (see section below) in the field. We then collected 30 additional female *G. affinis* (both years) and placed them in breathable bags to bring them back to the laboratory for behavior and life-history analysis. At each site, we obtained a point measure of water temperature, pH, salinity, conductivity, and total dissolved solids (plus nitrates in 2019), using hand-held water quality meters (YSI Inc.).

Measuring water-borne cortisol release rates

We measured individual cortisol release rates from *G. affinis* via a non-invasive water-borne hormone technique (following Scott & Ellis, 2007; Blake et al., 2014). We placed each collected individual into sterile 250 mL beakers containing 100 mL of spring water. Each beaker contained a LDPE plastic liner with holes on the bottom to easily transfer fish between beakers for repeated measures. Each fish remained in their beaker for 30 mins to obtain baseline cortisol release rates. Following 30 mins, 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 mins to obtain acute stress-response cortisol release rates. We also measured post-agitation cortisol recovery rates (2019 only) by moving the fish to a third sterile 250 mL beaker with 100 mL of spring water and allowed the fish to remain in the beaker for 1 hour. We then euthanized each fish by placing them in an ice-water slurry for 20 mins. We transferred water samples to

individual HDPE sample cups and stored them on ice. We then measured the standard length (SL) of each fish to the nearest tenth of a millimeter using dial calipers. We measured mass to the nearest centigram using a digital scale and then stored the fish in 70% ethanol for life-history analysis. Once in the laboratory, we stored water-borne hormone samples at -20 °C for future processing.

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 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. Final cortisol values (pg/ml) were multiplied by the total resuspension volume (0.720 ml), divided by SL, and multiplied by 2 for final unit of pg/mm/h. Cortisol EIA kits to assay water-borne hormone cortisol was previously validated by Blake et al. (2014) for *Gambusia geiseri*, a closely related species. We used a pooled sample of cortisol from non-experimental fish as our control in quadruplicate on each of the 11 experimental plates. The cortisol assays have a range from 6.6 to 400 pg/ml and a sensitivity of approximately 35 pg/ml. Our inter-assay coefficient of

variation for the control sample was 11.34% and our intra-assay coefficients of variation ranged from 0.70% to 8.80%.

Behavior

In 2018, we housed 30 female *G. affinis* per population in 37.85 L tanks (15 fish per tank) containing gravel and air bubblers for 40-50 hours after collection. We kept fish on a 14L:10D cycle at 25 °C and fed them half a teaspoon of tropical fish flakes (TetraMin) once daily. Following 40-50 hours, we transferred 5 fish at a time into a separate 37.85 L tank (50.8 cm x 25.4 cm x 30.5 cm) covered on all sides with dark-tinted glass, and let them acclimate for 10 mins. The tank was filled with dechlorinated water, approximately 5 cm from the bottom to restrict vertical column movement. After 10 minutes of acclimation, we remotely filmed fish from above for a total of 10 mins with a 1.3MP webcam (Dynex Inc.). After the 10 min trial time, we euthanized each fish in an ice-water slurry. We then measured the SL (mm) of each fish using dial calipers, and also measured mass (mg) on a digital scale. We then stored individual fish 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: inactivity time (s) and distance moved (cm). We also quantified group shoaling behavior which included: distance between subjects (cm) and time spent within 2 cm of other subjects (s).

In 2019, we housed 30 female *G. affinis* per population in 37.85 L tanks (14 fish per tank) for 40-50 hours after collection. Fish were kept on a 14L:10D cycle at 25 °C and fed tropical fish flakes (TetraMin) once daily. Following 40-50 hours, we measured their individual behavior. To do this, we caught individual fish with a small net and

transferred them to an opaque container (9 cm x 9 cm x 18 cm) filled with dechlorinated water and containing a square cutout for a door (5 cm x 5 cm) that was hinged to the lid connected to fishing line. The container served as a refuge and was placed in the corner of a shallow opaque plastic white tub (52 cm x 35 cm) containing 8 cm of treated water to restrict vertical movement. A webcam was mounted above each tub to record trials. We allowed the fish to acclimate in the refuge for 5 mins, and then remotely opened the door by pulling on the fishing line from the other side of the room. We ended the trial 5 mins after the fish left the refuge, or if the fish did not leave the refuge after 10 mins of observation. Four separate trials were recorded at the same time. After recording individual behavior, we recorded shoaling behavior by transferring 4 fish into an opaque (29 cm x 16 cm) container filled with 6 cm of treated water and another webcam mounted above. We recorded shoaling behavior for 5 mins. After the 5 min trial time, we euthanized each fish in an ice-water slurry. We then measured the SL (mm) of each fish using dial calipers, and also measured mass (mg) on a digital scale. We then stored individual fish in 70% ethanol for life-history analysis. We used Ethovision XT to quantify individual behavior which included: latency to emerge, inactivity time (s), and distance moved (cm). Latency to emerge was measured as the log of the maximum time allowed for the fish to exit the container (10 mins), minus the log of the latency time it took for the fish to exit the refuge and stay out for at least 10 consecutive seconds in the novel environment. A higher value for latency to emerge may indicate that the fish expresses more risk-taking behavior by entering the novel environment sooner. We also quantified group shoaling behavior which included: distance between subjects (cm) and time spent within 2 cm of other subjects (s).

Life-history

For both years, we dissected each female *G. affinis* ($n \approx 50/\text{population}$), removed their broods, and calculated fecundity as the total number of eggs (stages 1-3) and embryos (stage 4 plus) per fish (following Haynes, 1995). We then dried the broods and eviscerated fish for 48 hours 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 fish. We calculated individual propagule 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.

Statistical analyses

We conducted a Geographic Information Systems (GIS) analysis of land cover within the subwatershed surrounding each stream using the NLCD 2011 in ArcMap 10.6.1. Land cover classes were determined for each 30 m pixel. The sixteen land cover types recognized by the NLCD were reclassified into three classes: Undeveloped, Developed, and Agriculture. The Undeveloped class included Barren Land, Deciduous Forest, Evergreen Forest, Mixed Forest, Shrub/Scrub, Grassland/Herbaceous, Woody Wetlands, and Emergent Herbaceous Wetlands; Developed included Developed Open Space, Developed Low Intensity, Developed Medium Intensity, and Developed High Intensity; Agriculture included Pasture/Hay and Cultivated Crops. We derived a quantitative measure of urbanization by calculating the percent of the Developed land cover class within the subwatershed surrounding each sampling site, and used this value as a categorical variable in our analyses. Developed land cover highly correlated with impervious surface cover (Jennings et al., 2004). Statistical analyses were run using R

version 3.5.2 (R Core Development Team). We used a principle component (PC) analysis on the correlation matrix of environmental factors (temperature, conductivity, salinity, total dissolved solids (TDS), pH, nitrates, and % developed land cover) to summarize dominant gradients of environmental variability among sampling sites (Fig. 3). PC analysis was done using singular value decomposition (“prcomp” function).

To examine how cortisol release rates varied across populations and treatments (baseline, agitation, and recovery), we used linear mixed effect models (LMM) (“lmer” function in the “lme4” package) with individual fish as the random factor to account for repeated measures. All cortisol values were natural log transformed to meet assumptions of normality and homoscedasticity. We applied general linear models to examine differences in female SL, dry eviscerated mass (dry mass), fecundity, total RA, individual propagule dry mass, and behavior traits across a gradient of urbanization. Because fecundity is representative of count data, analyses for fecundity used generalized linear models (GLM) that were modeled with a Poisson distribution with a log link function (“glm”). Standard length, dry mass, total RA, individual propagule dry mass, and behavior traits were square-root transformed to meet assumptions of normality and homoscedasticity. Models for life-history included the eviscerated dry mass of the fish as a covariate to account for variation in body size. Models for behavior included the fish’s SL as a covariate to account for variation in body size. We used linear regressions (LR) to determine how urbanization (% development) influenced life-history trade-offs between fecundity and individual propagule dry mass. For both years, we also used general linear models with baseline and stress-response cortisol release rates as additional explanatory variables to examine how the components of GC regulation influenced life-

history traits (fitness) across a gradient of urbanization. In 2019 we also tested how the capacity to cope by rapidly responding (responsiveness) to and recovering (recovery) from a stressor affects fitness. We calculated responsiveness, the ability to respond to an acute stressor, as the difference between agitation and baseline cortisol release rates. We calculated recovery, the ability to sustain an acute stressor and return back to baseline, as the difference between agitation and recovery cortisol release rates. When appropriate, we used Tukey's *post hoc* tests to compare the means of our response variables across populations. To facilitate interpretation of the results, we conducted an equivalent set of linear models on untransformed data to generate graphs and least-square means in the original scale of the variables.

Results

Differences in cortisol across land development

In 2018, we found that more urbanized populations (25.4%, 51.3%) had higher cortisol release rates than the more rural populations (0.5%, 1.3%) and elicited a higher acute stress response to agitation (LMM: % Developed Land x Treatment; $F_{3,76} = 7.54$, $p < 0.0001$; Fig. 4a). All populations showed a significant stress response to agitation ($p < 0.05$), except at 1.3% development ($p > 0.05$). In 2019, we found that populations also differed in their cortisol release rates across development (LMM: % Developed Land; $F_{3,65} = 3.37$, $p = 0.0236$; Fig. 4b). Fish from the 25.4% developed site had the highest cortisol release rates, whereas fish from 5.2% developed site had the lowest cortisol release rates. Cortisol release rates also differed across treatments (LMM: Treatment; $F_{2,129} = 53.94$, $p < 0.0001$), with all populations showing a significant stress response to agitation ($p < 0.05$) but none of them recovering completely back to baseline ($p > 0.05$).

Life-history

In 2018, female fish differed in SL (ANOVA: % Developed Land; $F_{3,199} = 5.16$, $p = 0.0019$) and dry eviscerated mass (ANOVA: % Developed Land; $F_{3,199} = 7.20$, $p = 0.0001$) depending on how much developed land there was in the surrounding subwatershed (Table. 2). More urban streams had larger fish (Table 2). In 2019, female fish also differed in SL (ANOVA: % Developed Land; $F_{3,177} = 10.72$, $p < 0.0001$) and dry eviscerated mass (ANOVA: % Developed Land; $F_{3,178} = 14.82$, $p < 0.0001$) depending on how much developed land there was in the surrounding subwatershed (Table. 3).

In 2018, the relationship between female dry mass and fecundity significantly depended on the percent developed land cover in the surrounding subwatershed (GLM: % Developed Land x Dry Mass; $F_{3,187} = 3.00$, $p = 0.0319$; Fig. 5a). Each population showed a moderately strong positive relationship between female dry mass and fecundity. Fish from the most developed population (51.3%) had the highest fecundity for both large and small females, whereas fish from the least developed population (0.5%) had the lowest fecundity for both large and small females. While the relationship between dry mass and fecundity was similar for females from 1.3% and 25.4% developed sites in smaller fish, females from the 1.3% developed site had higher fecundity than females from 25.4% developed site when they were larger (Fig. 5a). In 2019, the relationship between female dry mass and fecundity also significantly depended on the percent developed land cover in the surrounding subwatershed (GLM: % Developed Land x Dry Mass; $F_{3,143} = 2.69$, $p = 0.0486$; Fig. 5b). Each population showed a moderately strong positive relationship between dry mass and fecundity. Fish from the most urban population (51.3%) had on average the highest fecundity. Fish from the least urban population (5.2%) had the lowest

fecundity when females were small. Fish from the 25.3% developed site had the largest fecundity when females were small. However, fish from 5.2% and 25.3% developed sites had similar fecundity when females were large (Fig. 5b).

In 2018, the relationship between female dry mass and total RA significantly depended on percent developed land cover (ANCOVA: % Developed Land x Dry Mass; $F_{3,183} = 4.26$, $p = 0.0062$; Fig. 6a). Each population showed a moderately strong positive relationship between dry mass and total RA. Fish from the most urban population (51.3%) had low RA when females were small, but the highest RA when females were large. Fish from the least urban population (0.5%) had a lower RA even when the females were large (Fig. 6a). In 2019, the relationship between female dry mass and total RA also significantly depended on percent developed land cover (ANCOVA: % Developed Land x Dry Mass; $F_{3,143} = 5.80$, $p = 0.0009$; Fig. 6b). Each population showed a moderately strong positive relationship between dry mass and total RA with the exception of the 25.4% developed site, which had a positive yet weak relationship. Fish from the most urban population (51.3%) had low RA when females were small, but had the highest RA when females were large (Fig. 6b).

In 2018, the life-history trade-off between individual propagule mass and fecundity significantly depended on the percent developed land cover (LR: % Developed Land x Fecundity; $F_{3,182} = 3.58$, $p = 0.015$; Fig. 7a). Fish from the 51.3% and 1.3% developed sites had a very weak negative relationship between individual propagule dry mass and fecundity, while fish from 25.4% and 0.5% developed sites had much stronger negative relationships between individual propagule dry mass and fecundity indicating a stronger trade-off (Fig. 7a). In 2019, all populations exhibited a significant negative

relationship between individual propagule dry mass and fecundity (LR: Fecundity; $F_{1,145} = 10.78$, $p = 0.0013$), such that individual propagule size decreased as fecundity increased (Fig. 7b). There were no significant differences in the magnitude of the trade-off across populations (LR: % Developed Land x Fecundity; $F_{3,142} = 1.44$, $p = 0.2332$), but there were significant differences in average individual propagule dry mass across populations (LR: % Developed Land; $F_{3,145} = 8.48$, $p < 0.0001$). Fish from the most rural population (5.2%) had the largest mean individual propagule dry mass.

GC-fitness relationships

In 2018, there were significant differences across populations in how the interaction between baseline cortisol release rates and female dry mass affected total RA (ANCOVA: % Developed Land x Baseline Cortisol x Dry Mass; $F_{3,60} = 7.27$, $p = 0.0036$; Fig. 8a-d). For the 0.5% and 51.3% developed sites, the highest RA was predicted in females that were both large and had high baseline cortisol release rates. For the 25.4% developed site, the highest RA was predicted in females that were large but had low baseline cortisol release rates. For the 1.3% developed site, the highest RA was predicted in large females, but baseline cortisol release rates did not appear to have any effect. There were no significant differences across populations in how the interaction between responsiveness and female dry mass affected total RA (ANCOVA: % Developed Land x Responsiveness x Dry Mass; $F_{3,60} = 0.63$, $p = 0.5968$).

In 2019, there were no significant differences across populations in how the interaction between baseline cortisol release rates and female dry mass affected total RA (ANCOVA: % Developed Land x Baseline Cortisol x Dry Mass; $F_{3,45} = 0.66$, $p = 0.5793$). However, there was a significant interaction between % development,

responsiveness, and recovery on total RA (ANCOVA: % Developed Land x Responsiveness x Recovery; $F_{3,41} = 3.02$, $p = 0.0404$; Fig. 9a-d). For the 5.2% and 25.4% developed sites, the highest RA was predicted in females that had weak responsiveness and strong recovery. For the 21.3% developed site, the highest RA was predicted for females that had strong responsiveness but weak recovery. For the 51.3% developed site, the highest RA was predicted for females that had both weak responsiveness and weak recovery.

Behavior

In 2018, there were significant differences among populations in individual inactivity time (ANCOVA: % Developed Land; $F_{3,118} = 7.66$, $p = 0.0001$; Fig. 10a). Fish from the most urban population (51.3%) spent less time moving. The relationship between female SL and their distance moved significantly depended on the percent developed land cover in the surrounding watershed (ANCOVA: % Developed Land x SL; $F_{3,115} = 3.39$, $p = 0.0205$; Fig. 11a). Fish from least developed site (0.5%) tended to explore the least. Fish from the 25.4% developed site explored the most at smaller sizes, but explored much less at larger sizes. There were no differences in group shoaling distance (ANOVA: % Developed Land; $F_{3,21} = 0.01$, $p = 0.9610$; Fig. 12a), nor group shoaling proximity (ANOVA: % Developed Land; $F_{3,21} = 0.12$, $p = 0.9500$; Fig. 13a) across populations.

In 2019, there were no significant differences across land development in inactivity time (ANCOVA: % Developed Land; $F_{3,40} = 1.25$, $p = 0.3060$; Fig. 10b), nor latency to emerge (ANCOVA: % Developed Land; $F_{3,36} = 0.53$, $p = 0.6667$). However, the distance individuals moved significantly depended on percent developed land cover

(ANCOVA: % Developed Land; $F_{3,40} = 4.30$, $p = 0.0102$; Fig. 11b). Fish from the 25.4% developed site explored the least, whereas fish from the 21.3% developed site explored the most. There were also significant differences in group shoaling distance (ANOVA: % Developed Land; $F_{3,22} = 6.15$, $p = 0.0034$; Fig. 12b), and group shoaling proximity (ANOVA: % Developed Land; $F_{3,22} = 6.27$, $p = 0.0031$; Fig. 13b). Fish from the 25.4% developed site shoaled closer together and for a longer period of time compared to the other populations.

Discussion

Understanding how organisms respond to environmental perturbations is becoming increasingly important as urban areas continue to expand. Species respond to urbanization in different ways, where some are able to adapt to urban living and others perish (Karr, 1981; Wang et al., 2001; Walsh et al., 2005; Santangelo et al., 2018). Those that can cope with urban perturbations and adjust their phenotypes to match their environment are better able to colonize and persist in urban areas (Sol & González-Lagos, 2013; French et al., 2018; Ouyang et al., 2019). We found that urban land development significantly influenced phenotypic differences in GC release rates, life-history traits, and behavior across populations of *Gambusia affinis*, a species of viviparous fish that commonly thrives in urban areas (Lloyd, 1986; Pyke, 2008). Our findings indicate that *G. affinis* are constantly responding to changes in their environment, and adjusting their phenotypes in ways that help them to persist in urban habitats.

Gambusia affinis varied in life-history traits across populations, years, and land development. In 2018, fish from more urban environments were larger than those from

less urban environments as represented by SL and dry mass. Although this pattern was not as strong in 2019, fish from the most urban population still had the greatest SL and dry mass. Female reproductive output positively covaried with female dry mass across populations and years, and the magnitude of these relationships depended on the percent developed land cover. Across both years fecundity was higher in females from more urban environments, and fish from the most urban population (51.3%) had the highest number of eggs and embryos. In 2018, female RA was generally higher in more urban populations. The most urban population (51.3%) had the highest RA and the least urban population (0.5%) had the lowest RA. In 2019, the most urban population (51.3%) had the highest RA in larger females, but RA was similar across populations in smaller females. Our results support the findings of Stearns (1983), where *G. affinis* produced more eggs and had higher reproductive investment in streams with higher water fluctuations, congruent with the hydrological changes that occur in urban streams. Similarly, Bennett et al. (2016) found that female *Cyprinella lutrensis*, another common invasive fish, had bigger clutch sizes in streams with greater and more variable flow regimes. Moreover, urban streams typically have warmer water temperatures and greater diurnal temperature fluctuations than rural streams (Paul & Meyer, 2001; Walsh et al., 2005), which can result in accelerated growth and increased brood mass in *G. affinis* (Vondracek, 1988). Although we found similar water temperatures across our sampling sites, we could not account for minimum, maximum, and temperature fluctuations as we only sampled at one time point early in the day. It is possible that the temperature regimes associated with urbanization influenced the life-history differences across populations.

There was a negative relationship between individual propagule mass and fecundity across populations and years, but the magnitude of this trade-off depended on the percent developed land cover. Across both years the trade-off between propagule size and number was the weakest in the most urban population (51.3%), and this population had on average the smallest propagules. Whereas the two least urban populations per year sampled (0.5% in 2018; 5.4% in 2019) had the strongest trade-off between propagule size and number, and females from the 5.4% developed site had the largest propagules in 2019. These results suggest that individual *G. affinis* that maximize their fitness by investing their energy into having more offspring, balance this energetic demand by having offspring of smaller size. The magnitude of this trade-off across environments may depend on outside ecological factors such as resource availability (Johnson & Bagley, 2011). For example, urban streams often exhibit an increase in algal biomass and surface prey (terrestrial insects), both of which are heavily consumed by *G. affinis* (Daniels & Felley, 1992; Mansfield & McArdle, 1998; Walsh et al., 2005). If urban streams have more algae, and storm water drainage systems wash more surface prey into the streams, then the compensatory life-history trade-off of increased offspring number to decreased offspring size may be mitigated by this increased energy food supply. By contrast, if food is limited, as in the oligotrophic rural habitats, then there should be a greater shift towards fewer yet larger offspring (Reznick & Yang, 1993), as seen in the more rural populations.

Changes in stream habitat quality and increased frequency of perturbations associated with urbanization may stress *G. affinis* and activate physiological coping mechanisms, helping them persist and maintain reproductive output despite the

challenges. The frequency and duration of challenges may vary from year to year, and could result in altered or dysregulated coping mechanisms in populations facing frequent or prolonged stressors. We found that cortisol release rates were generally higher in more urban populations, and all but one population were able to elevate their cortisol in response to an acute stressor (agitation), indicating proper functioning of the HPI axis in those populations. The population that failed to mount a stress response no longer had any *G. affinis* when surveyed in 2019, suggesting that they were not able to successfully cope with their environment. Together, our results fit our schematic predictions (Fig. 1a) that populations in more urban (and likely perturbed) sites would have higher cortisol levels.

Our study suggests that, in *G. affinis*, the capacity to cope with perturbations and regulate reproductive output is mediated by various components of GC regulation in a temporal and context specific manner. In 2018, baseline cortisol release rates increased with increasing urban land development as predicted (Fig. 1a). The most urban population (51.3%) and the most rural population (0.5%) had the highest RA in females that had high baseline cortisol release rates. These findings are congruent with the GC-Adaptation Hypothesis (positive relationship between baseline GCs and fitness), and parallel with other studies that have found baseline GCs increasing to support energetic demand during peak periods of reproductive investment (Romero, 2002; Bókonyi et al., 2009; Bonier et al., 2009; Patterson et al., 2014). For example, Bonier et al. (2009) found that female *Tachycineta bicolor* with higher baseline GCs had heavier clutch masses during periods of nestling provisioning. Similarly, Patterson et al. (2014) found that female *Zonotrichia leucophrys oriantha* with higher baseline GCs had greater

reproductive success. In the 25.4% developed population, highest RA was observed for females that had low baseline cortisol release rates. This result supports the GC-Fitness Hypothesis, suggesting that individuals or populations with elevated baseline GCs are facing greater challenges and have lower fitness than those with lower baseline GCs (Bonier et al., 2009). RA was similar for both high and low cortisol release rates in the 1.3% developed population, the same population that was unable to mount a stress response to agitation. There was no relationship between stress responsiveness and RA in 2018, and we did not measure recovery that year.

In 2019, we were better able to measure capacity to cope and how that related to fitness because we included a measure of recovery. Unlike in 2018, we did not find any relationships between baseline cortisol release rates and RA across populations sampled in 2019, though the least urban population (5.4%) had lower baseline cortisol release rates than the other three more urban populations. However, there were significant differences in how the interaction between responsiveness and recovery influenced RA across populations. In the 5.2% and 25.4% developed populations, highest RA was observed in females that had a trade-off between responsiveness and recovery. In particular, individuals with the highest RA had weak responsiveness but strong recovery. The opposite was observed in the 21.3% developed population, where RA was the highest in females that had strong responsiveness but weak recovery. Together, these findings support our predictions of what may occur in a moderately perturbed environment (Fig. 1b). For the most urban population (51.3%), highest RA was observed in females that had both weak responsiveness and weak recovery. This finding supports our prediction for the relationship between coping capacity and fitness in a highly

perturbed environment (Fig. 1b), and indicates that there is a cost to responding to stressors and recovering in terms of fitness.

Evidently, baseline and stress-induced GCs all play different roles in coping with challenges, depending on the severity and stochasticity associated with the environment. Our predictions regarding GC-fitness relationships generally matched our results, though not always. The regulation of baseline GCs influenced RA in 2018 but not in 2019. A possible explanation for this finding is that 2019 had almost twice as much rainfall as 2018, which may have increased the frequency and duration of perturbations related to hydrology, water quality, and predation. These increased perturbations could result in more frequent activation of the HPI axis and therefore more frequent cascades of responsiveness and recovery in an attempt to cope with the stressors and return to homeostasis. While baseline GCs regulate daily metabolic demand and increase in response to predictable seasonal changes such as timing of reproduction, stress-induced GCs regulate homeostasis and coordinate responses to unpredictable and severe changes in the environment (Wingfield & Kitaysky, 2002; Landys et al., 2006). Therefore, baseline GCs may influence fitness in more stable environments, whereas stress responsiveness and recovery GCs may influence fitness in more stochastic and stressful environments. Furthermore, Kim et al. (2018) found that cortisol release rates in a laboratory population of *Poecilia latipinna*, another species of Poeciliid fish, did not change with increasing reproductive effort. Therefore, we can surmise that GCs mediate fitness in Poeciliid fish, and are not simply elevated as an artifact of increased reproductive demand.

Although percent impervious surface cover is an accurate predictor of ecological degradation in urban streams, our sampling sites had unique differences in environmental factors such as food availability, water quality, water flow, population density, predation intensity, and pollutants which can all vary temporally and drive population-level differences in GC regulation, life-history, and behavior (Johnson & Bagley, 2011). Nevertheless, our results do generally support our predictions regarding the relationships between GCs and fitness. For example, in 2019, females from the most urban population (51.3%) had negative relationships between responsiveness and recovery with fitness, which supports the idea that organisms with flexible reproductive strategies would increase their sensitivity to environmental cues and likelihood of reproductive success in high perturbation environments (Lattin et al., 2016; Vitousek et al., 2019). Female *G. affinis* do indeed have a flexible reproductive strategy as they only need to mate once, can store sperm for a large portion of their lifetime, flexibly adjust when their eggs are fertilized, and give birth to live young (Haynes, 1993; Pyke, 2008). The moderately perturbed populations (21.3% and 25.4%) supported the predicted trade-offs between responsiveness and recovery with fitness. These two populations also had similar water quality characteristics (Fig. 3). The least disturbed population did not support the predicted positive relationship between responsiveness and recovery with fitness, but rather a trade-off as expected in a moderately perturbed environment. However, this population also had more agricultural land in its surrounding subwatershed (11%) than any other population, which could have driven the observed discrepancy in the predicted GC-fitness relationship.

Variation in the individual behavior of *G. affinis* depended upon developed land cover and sampling year. In 2018, fish from the most urban site (51.3%) spent less time moving compared to the three less urban populations, which could put them at greater risk for predation. Urban streams typically have lower fish species diversity (Paul & Meyer, 2001), suggesting that there may have been fewer predators present in the most urban population. A lack of predators could favor more sedentary behavior in *G. affinis*, as a means to conserve energy. However, the average duration that fish moved did not differ among populations in 2019. In 2018, fish from the least urban site (0.5%) explored their novel environment less than the three more urban populations. This could imply that very rural populations of *G. affinis* do not disperse and colonize new territories as well as more urban populations (Cote et al., 2010). Additionally, individuals from this population may be less successful at competing for resources, which could also explain the small sizes of individuals in this population. In 2019, fish from the 25.4% developed site explored the least, whereas fish from the 21.3% developed site explored the most. There were no differences in latency to emerge in to a novel environment for fish sampled in 2019. Variation in the shoaling behavior of *G. affinis* also varied by land cover and sampling year. There were no difference in group shoaling distance nor group shoaling proximity for populations sampled in 2018. However, fish from the 25.4% developed site shoaled closer together and for a longer period of time compared to all other populations sampled in 2019. Shoaling is usually viewed as a defense mechanism against predators and also generally results in more efficient foraging (Pitcher, 1986; Laland & Williams, 1997). We can surmise that, this moderately developed population may have been exposed to more predators, and are also likely more efficient at foraging.

Understanding how wildlife populations cope with changing environments is increasingly important as urban areas continue to expand. Our findings demonstrate that urbanization alters the stress physiology, life-history, and behavior of *G. affinis*, and that their reproductive output is mediated by context-specific variation in GC regulation. These flexible traits show temporal variation across populations, but also differ predictably among populations as a result of local adaptations. These traits may be the biomarkers that should be considered when describing a fish as tolerant or not. Urban streams are generally unfavorable to many native and intolerant fish species, yet *G. affinis* have been able to adapt by regulating their GC physiology in favor of larger broods in these perturbed environments. While baseline GCs may be important for maximizing fitness during predictable seasons, stress-response and recovery GCs may be more important in more stochastic seasons, where the magnitude and duration of perturbations are more severe. Using GCs as biomarkers to predict fitness and behavior of *G. affinis*, and other fish, to changing environments can help researchers and land managers better evaluate stream health, and inform on the mechanisms driving community structure in land-use converted areas.

TABLES

Table 1 | Description of the sampling sites. Land cover classes are based on the subwatershed area surrounding the sampling site. We focus on % developed land cover because it highly correlates to impervious surface cover, which is an accurate indicator of urbanization.

Population	Years Sampled	Undeveloped Land (%)	Developed Land (%)	Agricultural Land (%)
Bull Creek	2018, 2019	73.45	25.38	0
Walnut Creek	2018, 2019	46	51.3	2.03
Long Branch Creek	2018	98.57	1.32	0
Lone Man Creek	2018	98.46	0.52	0
Onion Creek	2019	77.98	21.34	0.4
Blanco River	2019	82.78	5.24	10.95

Table 2 | Least square means and standard errors for standard length (SL) and eviscerated dry mass for fish sampled in 2018.

Population	Developed Land	SL (Mean \pm SE) (mm)	Dry Eviscerated Mass (Mean \pm SE) (mg)
Bull Creek	25.38 %	34.91 \pm 0.75	134.70 \pm 7.77
Walnut Creek	51.3 %	32.80 \pm 0.73	120.43 \pm 7.55
Long Branch Creek	1.32 %	30.91 \pm 0.75	84.70 \pm 7.77
Lone Man Creek	0.52 %	31.70 \pm 0.75	99.34 \pm 7.77

Table 3 | Least square means and standard errors for standard length (SL) and eviscerated dry mass for fish sampled in 2019.

Population	Developed Land	SL (Mean \pm SE) (mm)	Dry Eviscerated Mass (Mean \pm SE) (mg)
Bull Creek	25.38 %	26.47 \pm 0.79	71.33 \pm 8.73
Walnut Creek	51.3 %	30.62 \pm 0.79	125.39 \pm 8.92
Onion Creek	21.34 %	25.63 \pm 0.80	55.84 \pm 8.73
Blanco River	5.24 %	30.33 \pm 0.79	106.89 \pm 8.73

FIGURES

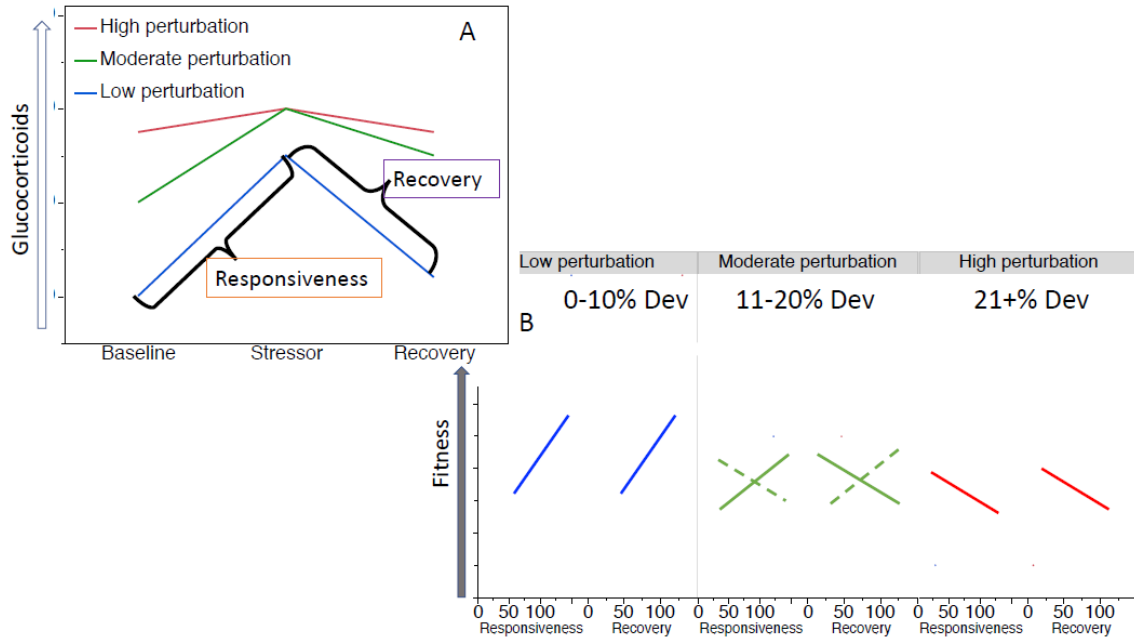


Fig. 1: Capacity to cope with environmental perturbations. (a) A schematic illustration of the putative glucocorticoid response to environmental perturbations. (b) Predicted relationships between glucocorticoid responsiveness and recovery with fitness.

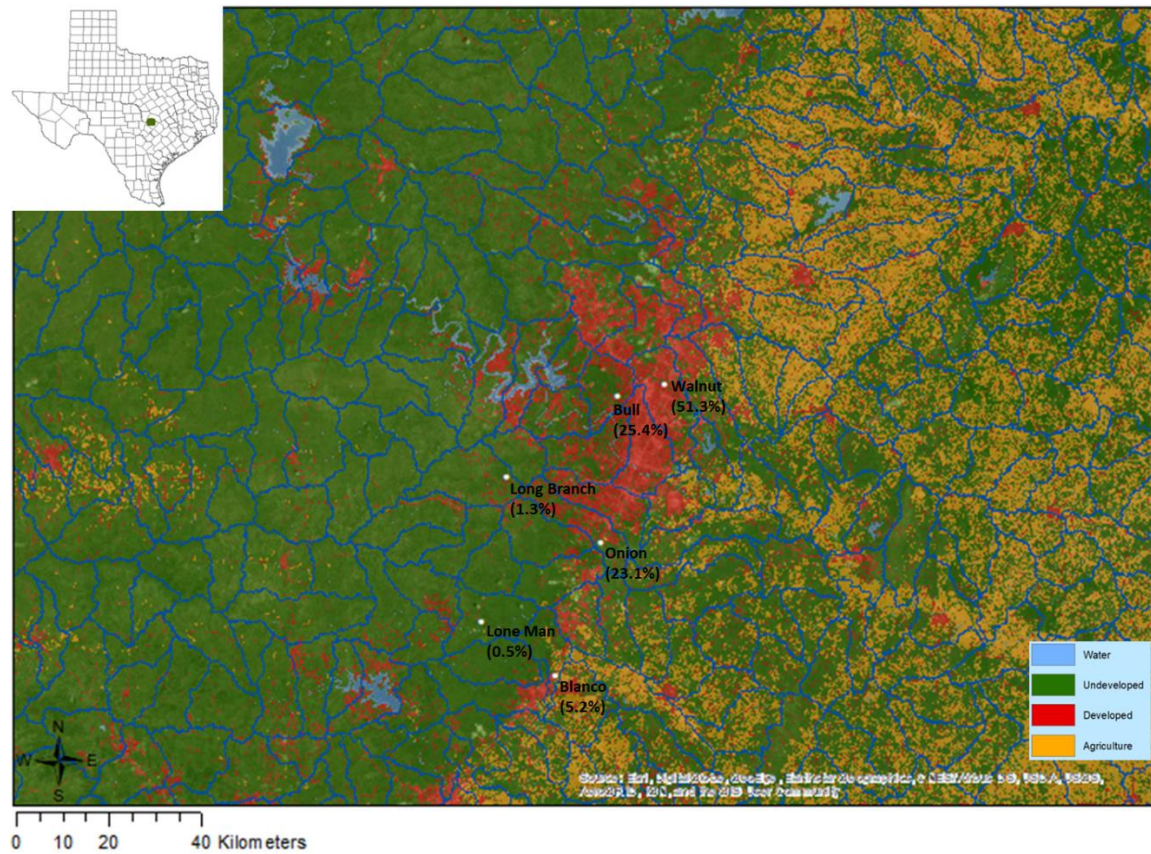


Fig. 2: Map of the six study areas in Central Texas, USA with their percent developed land cover in parenthesis.

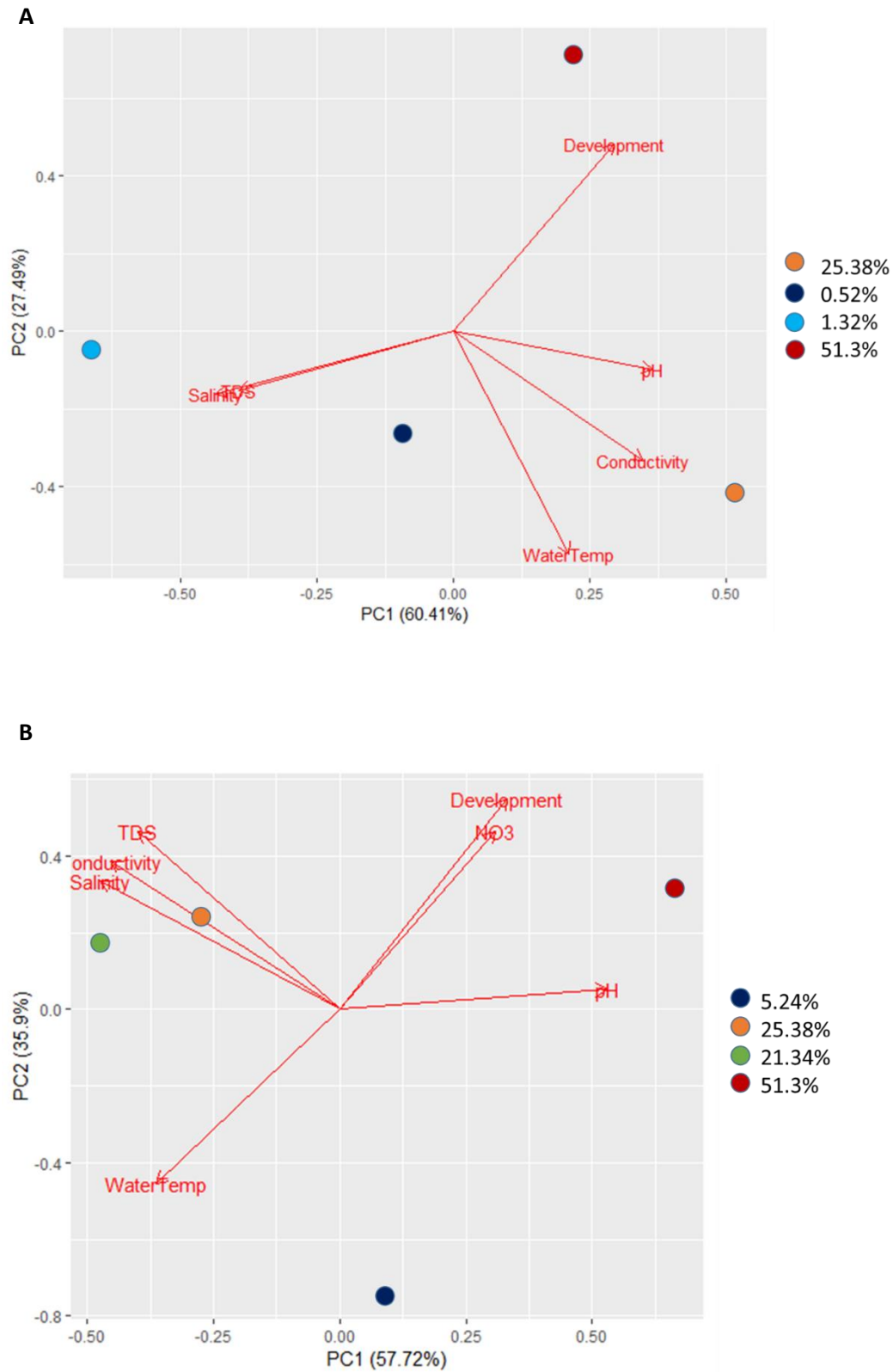


Fig. 3: Ordination plot from the principle components analysis of the *G. affinis* sampling sites based on (a) six habitat characteristics for 2018 and (b) seven habitat characteristics for 2019.

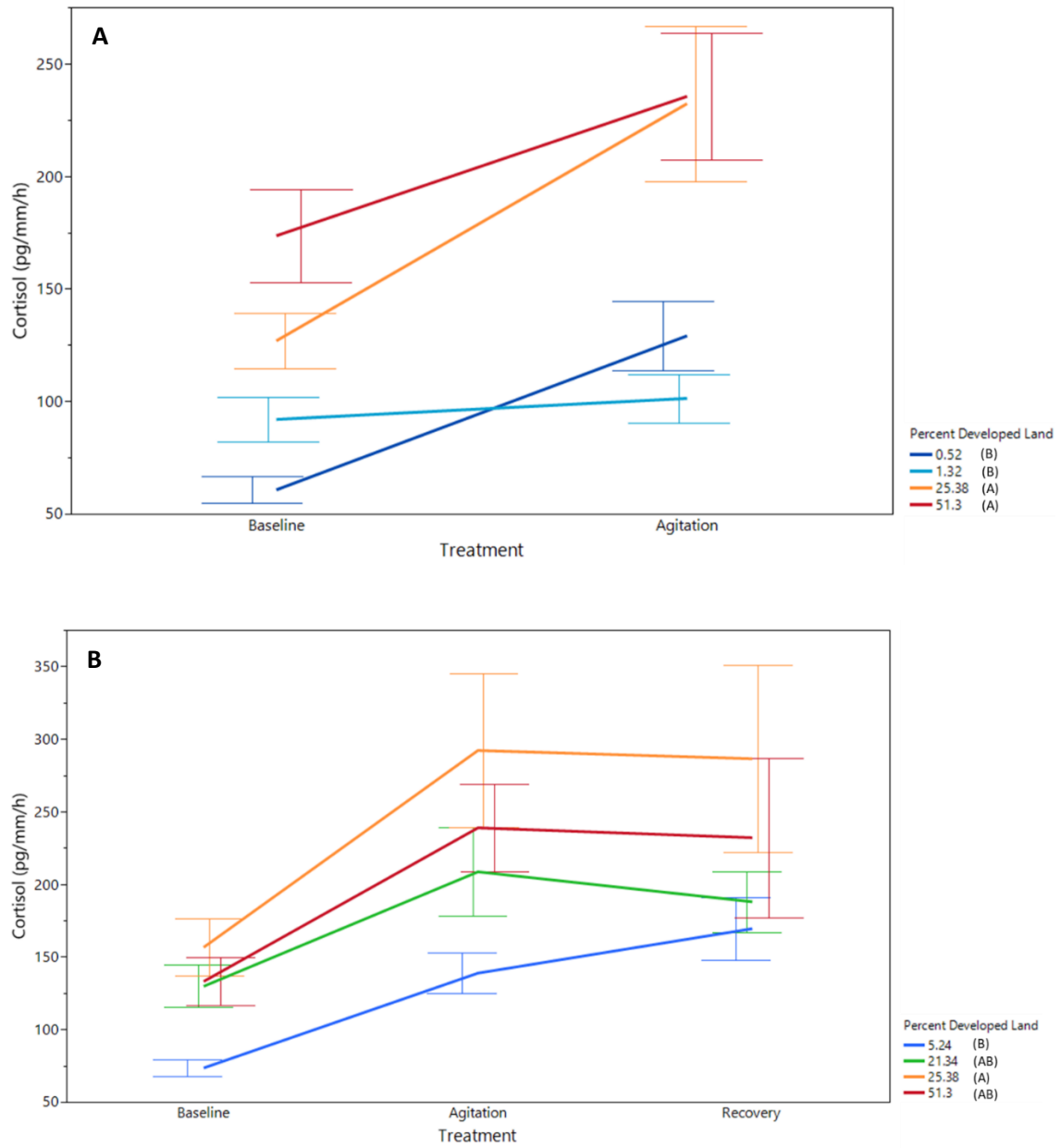


Fig. 4: Mean (\pm SE) cortisol release rates (pg/mm/h) of female *G. affinis* for each population (% Developed Land) and across (a) treatments: Baseline and Agitation from 2018 and (b) treatments: Baseline, Agitation, and Recovery for 2019. Letters next to legend indicate significant differences across populations.

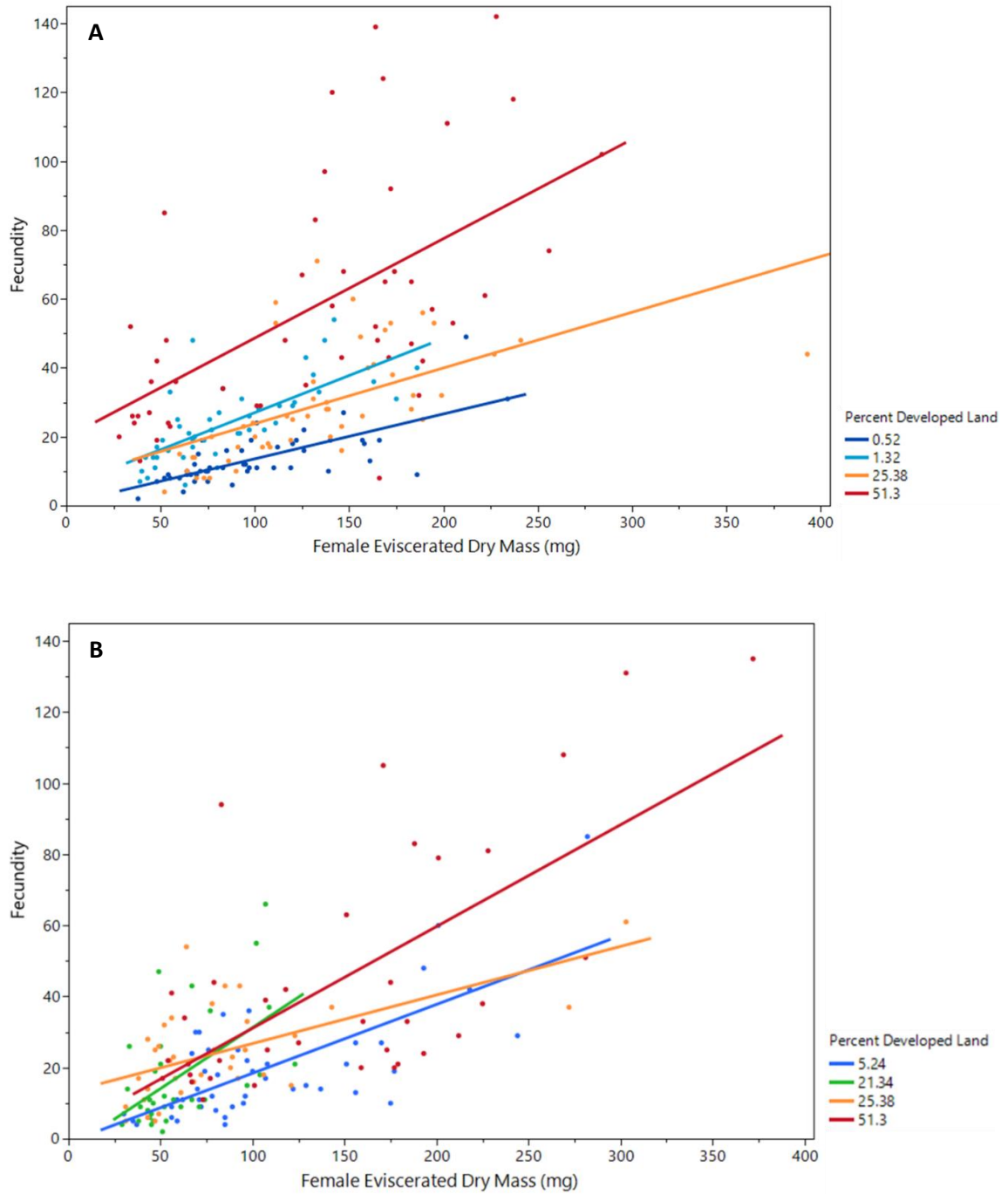


Fig. 5: The relationship between fecundity (eggs and embryos) and dry eviscerated mass (mg) for female *G. affinis* across populations sampled in (a) 2018 and (b) 2019.

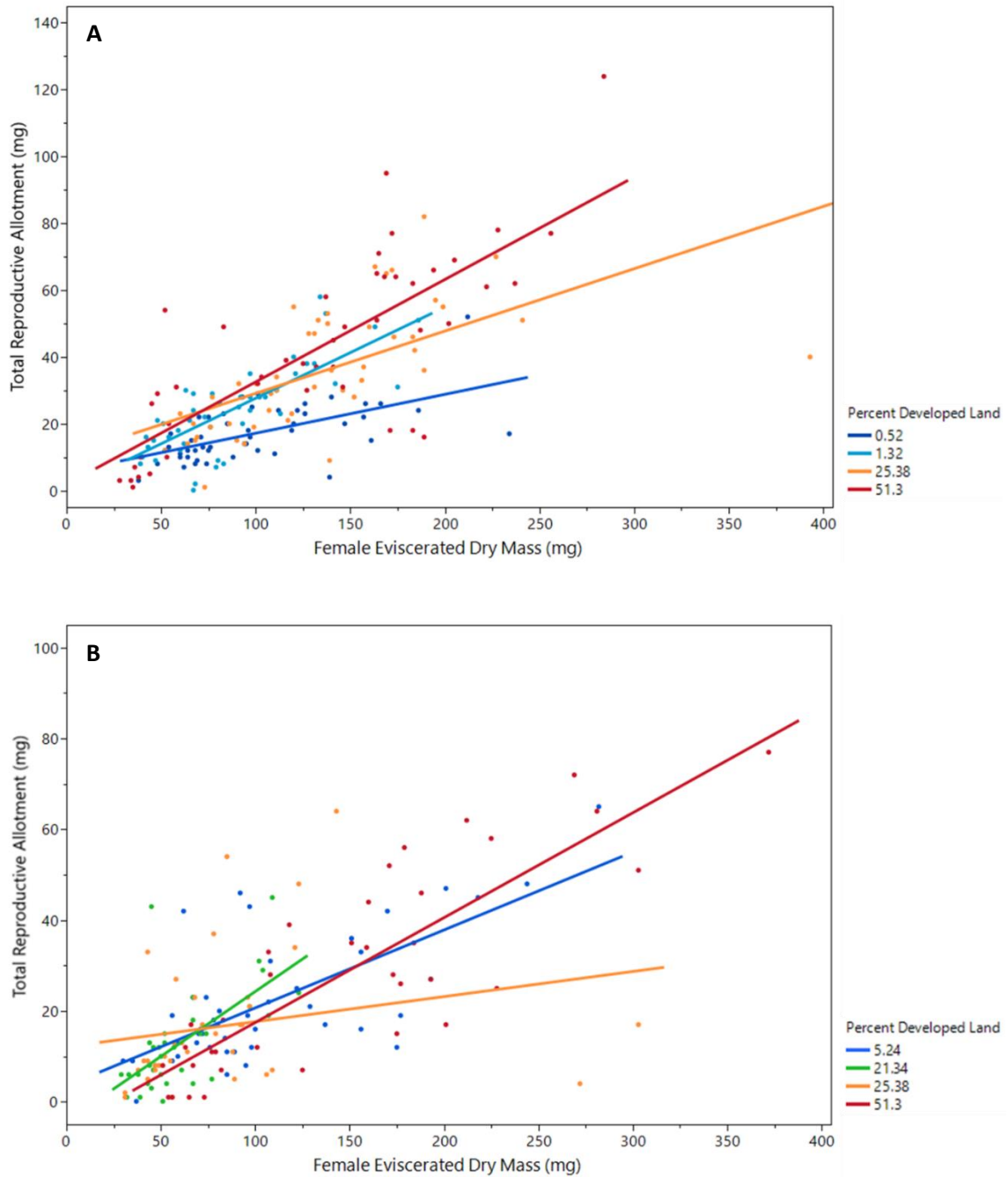


Fig. 6: The relationship between total RA (mg) and dry eviscerated mass (mg) for female *G. affinis* across populations sampled in (a) 2018 and (b) 2019.

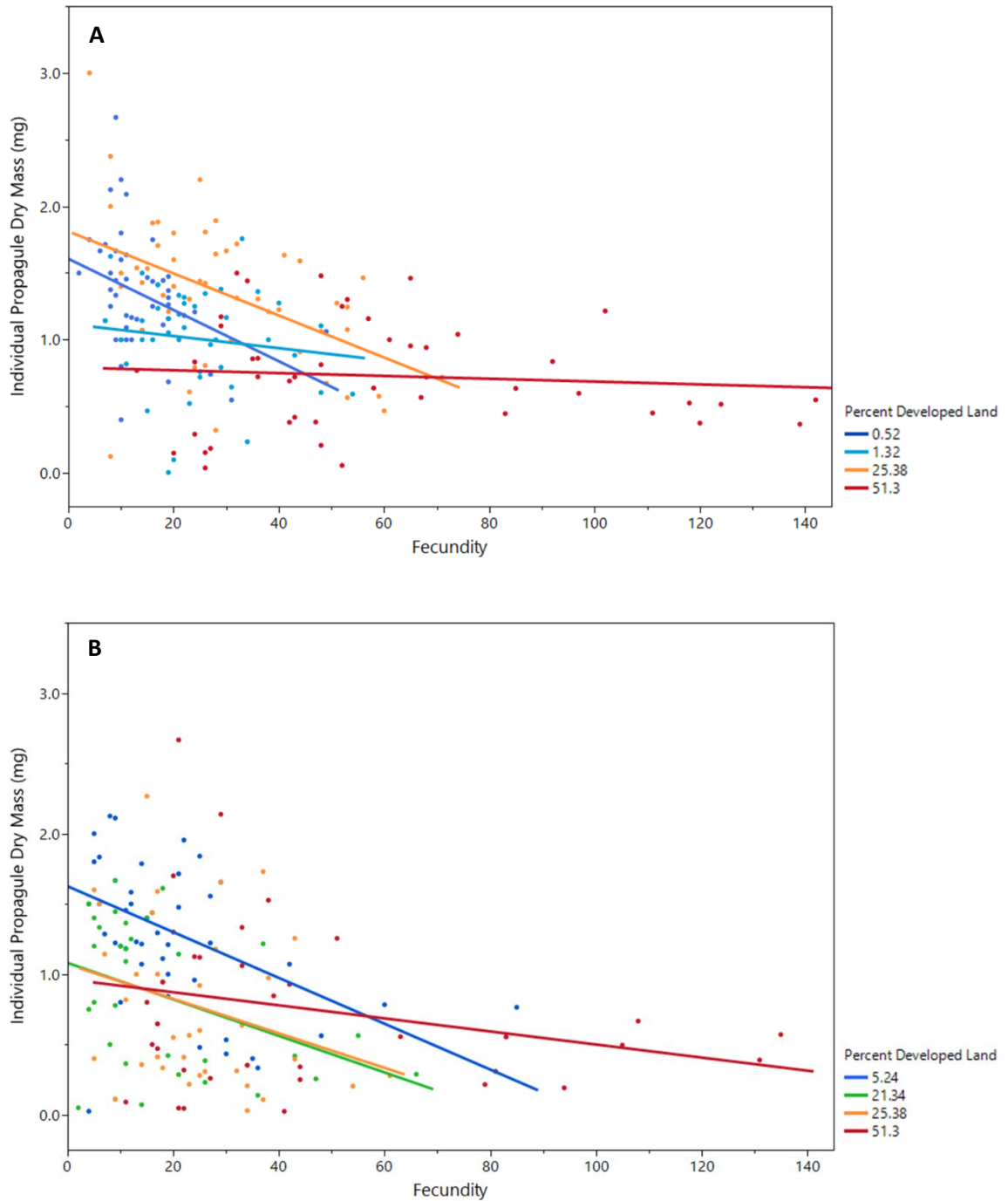


Fig. 7: The life-history trade-off between fecundity (eggs and embryos) and individual propagule dry mass (mg) for female *G. affinis* across populations sampled in (a) 2018 and (b) 2019.

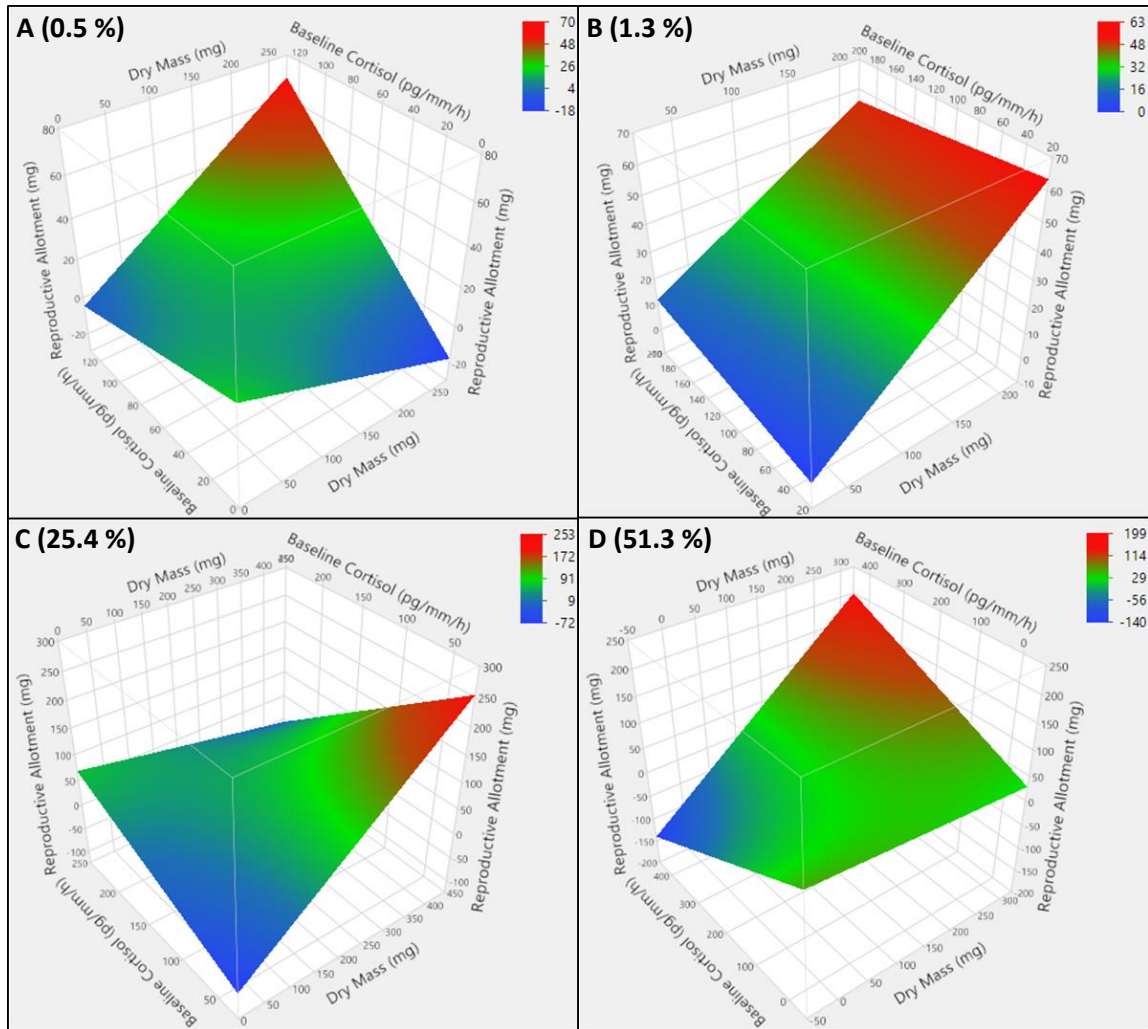


Fig. 8: Three-dimensional surface plots illustrating the relationships between total RA, baseline cortisol (pg/mm/h), and dry eviscerated mass (mg) of female *G. affinis* across populations sampled in 2018. Developed land cover: (a) 0.5% (b) 1.3% (c) 25.4% (d) 51.3%.

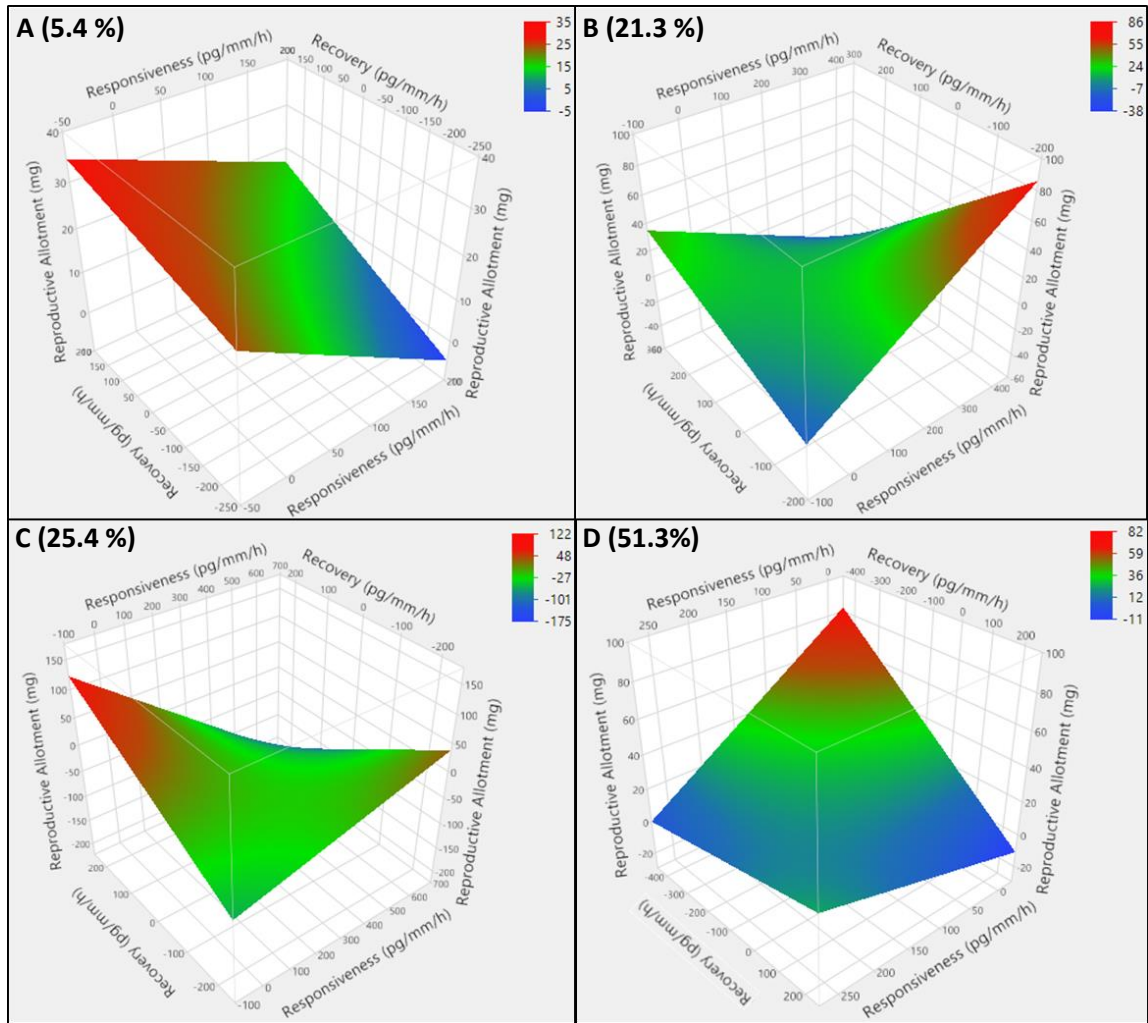


Fig. 9: Three-dimensional surface plots illustrating the relationships between total RA, stress-response cortisol (pg/mm/h), and negative-feedback cortisol (pg/mm/h) of female *G. affinis* across populations sampled in 2019. Developed land cover: (a) 5.4% (b) 21.3% (c) 25.4% (d) 51.3%.

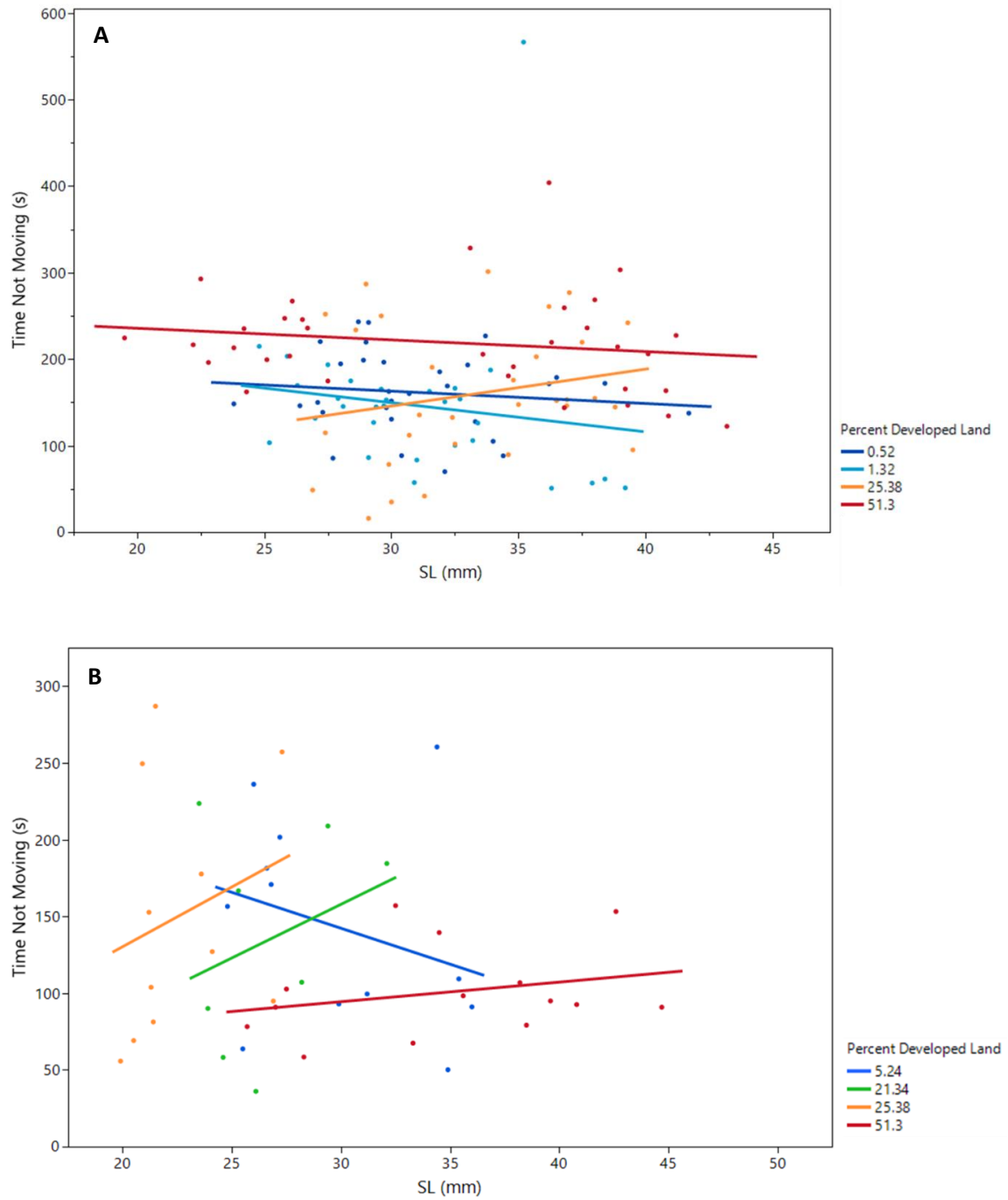


Fig. 10: The relationship between inactivity time (s) and SL (mm) for female *G. affinis* across populations sampled in (a) 2018 and (b) 2019.

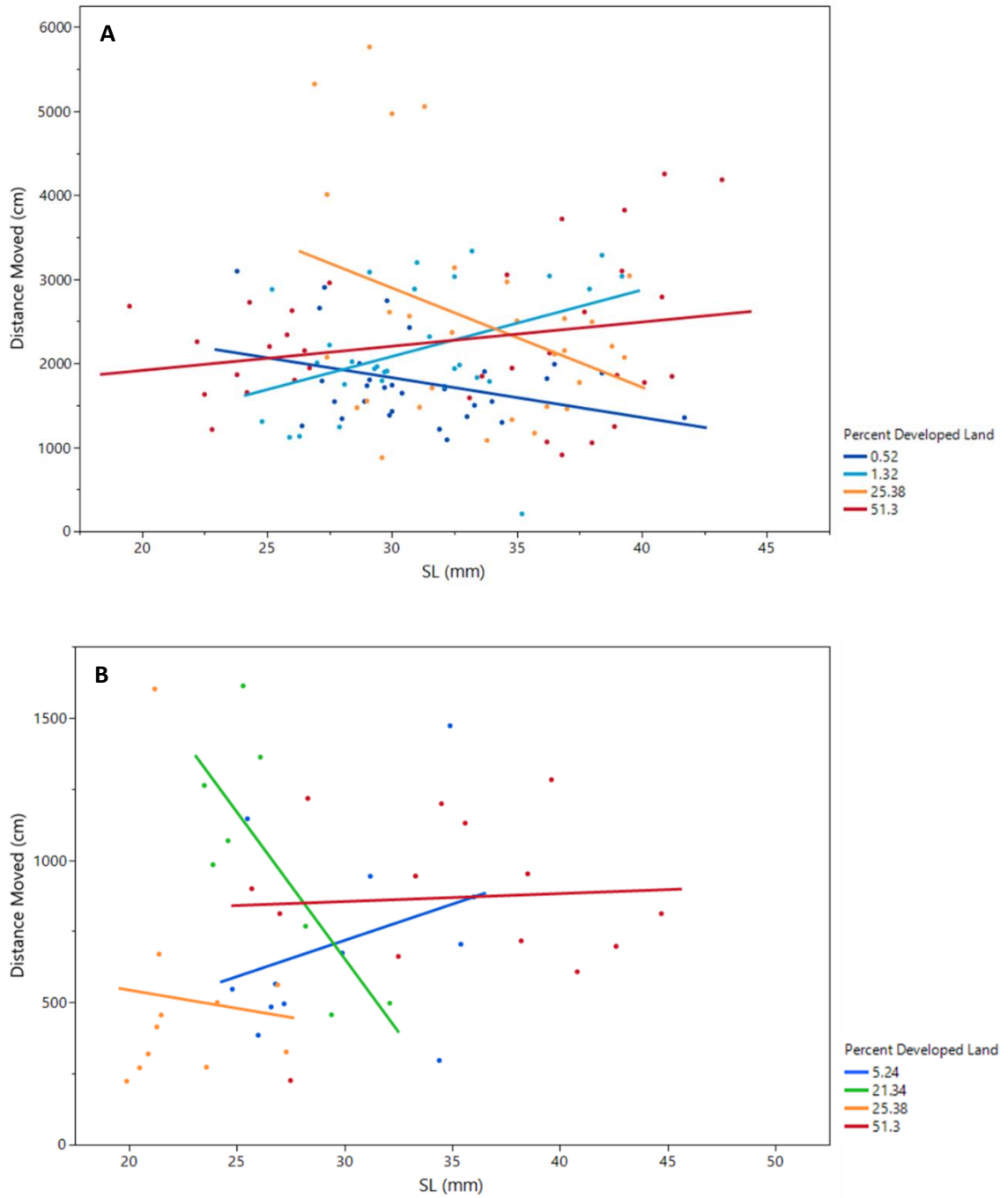


Fig. 11: The relationship between distance moved (cm) and SL (mm) for female *G. affinis* across populations sampled in (a) 2018 and (b) 2019.

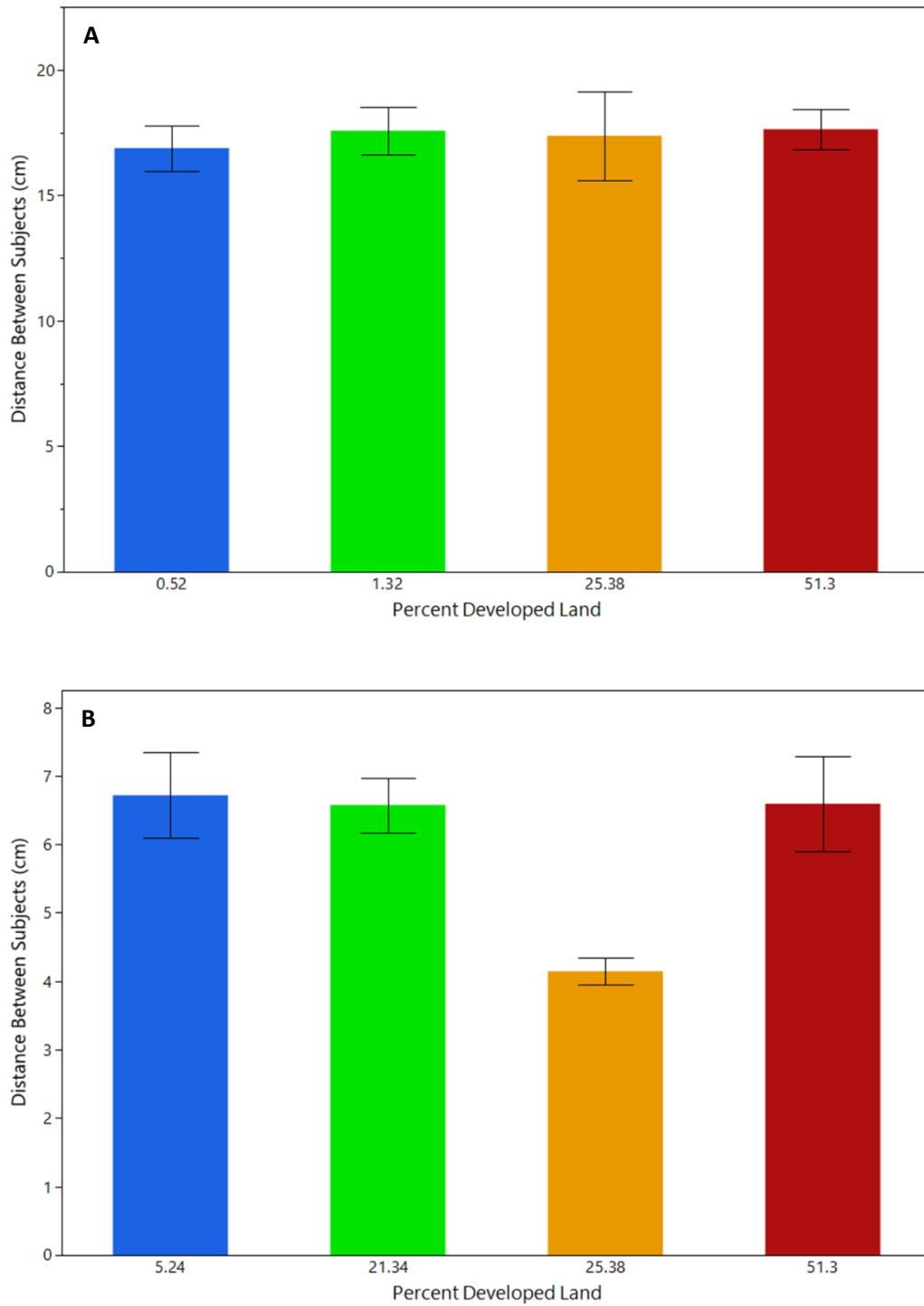


Fig. 12: Mean (\pm SE) distance between fish spent shoaling together for groups of female *G. affinis* across populations sampled in (a) 2018 and (b) 2019.

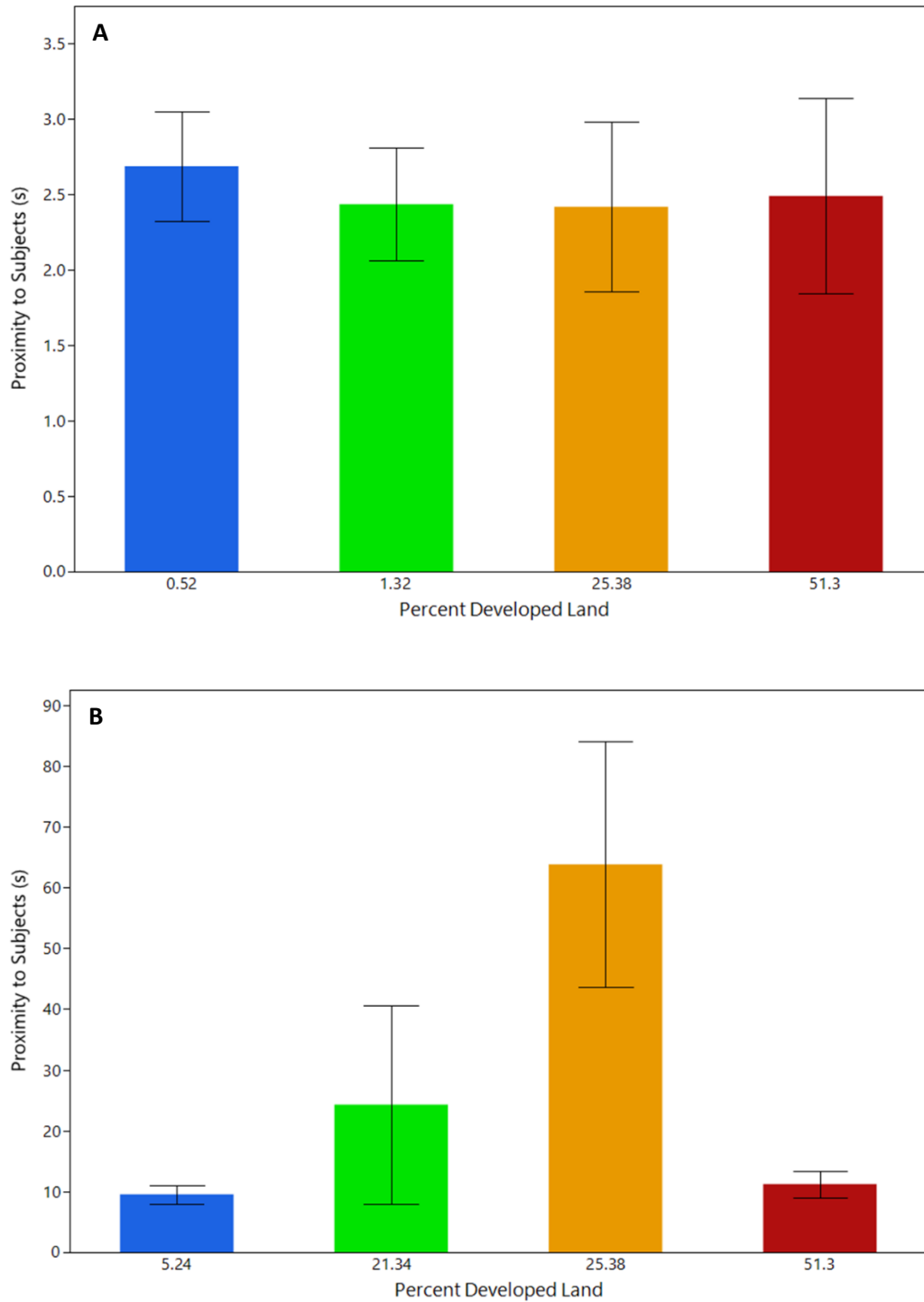


Fig. 13: Mean (\pm SE) time spent shoaling together for groups of female *G. affinis* across populations sampled in (a) 2018 and (b) 2019.

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