ARTIFICIAL LIGHT AT NIGHT ALTERS PHYSIOLOGY, BEHAVIOR, AND REPRODUCTION IN THE WESTERN MOSQUITOFISH (*GAMBUSIA AFFINIS*)

by

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A thesis submitted to the Graduate Council of
Texas State University in partial fulfillment
of the requirements for the degree of
Master of Science
with a Major in Population and Conservation Biology
May 2020

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ACKNOWLEDGEMENTS

I thank my advisor, Dr. Caitlin Gabor, for accepting me into her lab and guiding me along my graduate school journey in a myriad of ways. I also thank Dr. Mar Huertas, for her guidance in teaching me about fish physiology and how to perform laboratory techniques, and Dr. Andrea Aspbury for her mentorship in writing, statistics, and teaching.

I thank my fellow lab members and peers for their help and support both in and out of the lab. I am grateful for their friendship and for the countless times they have been there for me throughout the last couple of years. Thank you, Arseniy, Zach, Mel, McKenna, Cory, Alex, Amanda, Dillon, and many more.

I have extreme gratitude towards my previous lab at Boise State University. I thank them for their friendship and for giving me the opportunities and tools necessary to get where I am today. I thank Jesse Barber, Juliette, Brian, Eli, Mitch, Cory, Nic, Amanda, and many others. I would also like to thank the Pioneer Toastmasters group in Boise, Idaho for helping me overcome my fear of public speaking and for giving me invaluable lessons in leadership.

None of my accomplishments would be possible without the constant support of my family. I thank my parents, my sisters, my brother, my grandparents, and many aunts, uncles, cousins, nieces, and nephews.

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ABSTRACT

Human population growth and its associated effects have contributed to the rapid decrease of biodiversity worldwide. Artificial light at night (ALAN) is an anthropogenic pollutant that is increasing with the spread of urbanization and may contribute to biodiversity declines. ALAN alters the migration patterns of birds, communication in frogs, and impacts reproduction, behavior, and physiology of multiple other taxa. The effects of ALAN on freshwater organisms, however, are largely understudied as compared to terrestrial taxa. I investigated how ALAN affects the physiology, behavior, and reproduction of a widespread freshwater fish. *Gambusia affinis* are small livebearing fish often found in urban streams. I exposed groups of female *G. affinis* to either a natural light cycle or a constant 24-hour light cycle (ALAN) in the laboratory for 60 days. In another experiment, I exposed females to the same treatments in outdoor mesocosms for 32 days. I found that exposure to ALAN lowered glucose levels in brain tissue and decreased activity, but had no effect on cortisol release rates, reproduction, survival, or growth. This research is strengthened by measuring multiple metrics in response to ALAN and by incorporating both a field and laboratory component which confirm similar results. Ultimately, these results suggest that ALAN has detrimental effects even on a tolerant species and serious efforts should be taken to reduce its propagation.

I. LITERATURE REVIEW

In aquatic ecosystems, anthropogenic disturbances contribute to shifts in water quality (e.g. pollutants and added nutrients), changes in phenology, sound, and light pollution (Jenkins, 2003; Allan, 2004; Barbier, 2012; Davies *et al.*, 2014; Swaddle *et al.*, 2015; Shannon *et al.*, 2016; Buxton *et al.*, 2017; Sordello *et al.*, 2019). Such disturbances lead to rapid declines in biodiversity in aquatic ecosystems globally (Ceballos *et al*., 2015). Loss of biodiversity has a negative impact on ecosystem function and is increasing globally at a rate of 6% per year, leading earth into the sixth mass extinction (Butchart *et al*., 2010; Hölker *et al.*, 2010; Barnosky *et al*., 2011; Cardinale *et al*., 2012; Cellabos *et al*., 2015). Considering freshwater ecosystems, which contain 9.5% of all globally described species, models predict species declines to exceed those of tropical rainforests, primarily due to anthropogenic disturbances (Ricciardi & Rasmussen, 1999; Xenopoulos *et al*., 2005; Dudgeon *et al*., 2006; Balian *et al*., 2008). Empirically, the numbers of described North American fish species that have gone extinct increased 25% from 1989 to 2012 (Burkhead, 2012).

Artificial light at night (ALAN) is a form of pollution associated with anthropogenic disturbances (Longcore & Rich, 2004; Navara & Nelson, 2007). Light plays a key role in the ecology of organisms as a source of energy and information, a regulator of circadian rhythms, and as a cue for communication, navigation, and orientation (Gaston *et al.*, 2012). Aquatic organisms are affected by ALAN because they are influenced by photoperiod during all phases of their life history (Downing & Litvak, 2002; Mehner, 2012). I propose to study the effects of ALAN on the physiology,

reproduction, and behavior of the western mosquitofish, *Gambusia affinis*, a widespread livebearing fish (Lloyd, 1986).

Artificial light at night

Artificial light at night (ALAN) is artificial light that alters the natural light and dark cycle in an ecosystem (Swaddle *et al.,* 2015). Sources of ALAN include field lights, headlights, building lights, and lights associated with fishing boats and marine vessels. These sources accumulate to form skyglow, or light that is reflected back from the sky (Longcore & Rich, 2004). ALAN has become so widespread that ⅔ of the global human population lives under light-polluted skies and 40% lives in areas that are continually illuminated due to ALAN (Cinzano *et al*., 2001; Swaddle *et al.,* 2015). Furthermore, 90% of the global human population lives within 10 km of a body of water and 50% lives within 3 km, making freshwater and coastal marine areas the most impacted by anthropogenic disturbances (Dudgeon *et al.,* 2006; Kummu *et al.,* 2011; Venohr *et al.,* 2018). Yet, most studies involving ALAN focus on terrestrial taxa (Depledge *et al.,* 2010). These studies show that ALAN can have pervasive effects that alter ecological communities (Davies *et al.,* 2012; Gaston *et al.,* 2012; Becker *et al.,* 2013; Manfrin *et al.,* 2017; Czarnecka *et al.,* 2019; Maggi *et al.,* 2019).

ALAN has negative effects on many taxa. In humans ALAN reduces the production of melatonin (Lewy *et al.,* 1980) which is linked to performance, alertness, and sleep disorders, diabetes, obesity, depression, oxidative stress, heart disease, and certain cancers (reviewed in Navara & Nelson, 2007; Falchi *et al.,* 2011). ALAN has detrimental effects on wildlife that include reproduction, foraging, orientation, predation,

physiology, and migration (Longcore & Rich, 2004; Navaro & Nelson, 2007; Hölker *et al*., 2010**;** Ouyang *et al.,* 2011; Gaston *et al.,* 2013; Davies *et al*., 2014; Gaston *et al.*, 2014, 2015). For example, ALAN alters territorial singing and calling behavior in frogs, and communication in frogs, fireflies, and coyotes (Lloyd, 1994; Bergen & Abs, 1997; Baker & Richardson, 2006; Underhill & Höbel, 2018; Dias *et al.*, 2019). Additionally, ALAN reduces visual capability in frogs and attracts many insect families to artificial light sources (Buchanan, 1993; Eisenbeis & Hassel, 2000). Mate selection, nest selection, and timing of reproduction are also altered by ALAN in birds and frogs (Rowan, 1938; Rand *et al.,* 1997; De Molenaar *et al*., 2000). ALAN alters movement patterns and disrupts orientation in fish, turtles, insects, reptiles, birds, and amphibians (Witherington & Bjorndal, 1991; Buchanan, 1993; Salmon *et al.*, 1995; Evans-Ogden, 1996; van Langevelde *et. al.*, 2011).

Some species may benefit from exploiting ALAN, which extends opportunities for foraging and activity at night, as found in some reptiles, diurnal birds, insectivorous bats, and invertivore fishes (Schwartz & Henderson, 1991; Tabor *et al*., 2004; Schoeman, 2016). While some species may benefit from increased foraging time, they are also under increased predation risks while doing so (Gotthard, 2000). Yurk and Trites (2000) demonstrate that juvenile salmonids are more heavily preyed upon on by harbor seals under artificial lights than those in complete darkness. Many taxa forage more at low levels of illumination compared to high levels and avoid predation as a consequence (Gilbert & Boutin, 1991; Rydell, 1992; Lima, 1998; Moore *et al*., 2000; Tabor *et al.,* 2004). The majority of these studies are focused on terrestrial taxa and there is a paucity of data on how ALAN affects aquatic organisms, especially in freshwater (Depledge *et*

al., 2010; Perkin *et al.*, 2011). May *et al.*, (2019) found that wood frogs, *Lithobates sylvaticus*, show decreased activity and increased susceptibility to trematodes but had no effect on survival or time to metamorphosis after exposure to ALAN. Fish experience altered melatonin rhythms and activity when exposed to ALAN (Becker *et al.*, 2013; Brüning *et al.*, 2015; Sanders & Gaston, 2018; Czarnecka *et al.*, 2019) and zooplankton activity is also altered (Moore *et al.*, 2000). Reduced activity of zooplankton could lead to enhanced algal biomass which could lower water quality. These results indicate that ALAN has the potential to alter community structure in freshwater habitats.

Physiological response to stress

Hormonal systems provide mechanisms for organisms to adjust behavior, morphology, and physiology to changing environmental conditions (Zera *et al*., 2007). Therefore, the effects of environmental disturbances can be studied by analyzing the disruption of endocrine responses. Vertebrates secrete glucocorticoids (GC), primarily in the form of cortisol and or corticosterone, which mediate routine responses necessary to maintain homeostasis and may also increase in response to acute stressors (Sapolsky *et al*., 2000; Wingfield, 2013). The process of releasing GCs begins when the brain releases corticotropin-releasing hormone (CRH), a neuropeptide, which in turn stimulates secretion of adrenocorticotropin (ACTH) from the pituitary and acts on adrenocortical cells resulting in the production of GCs (Wingfield & Romero, 2001). To deactivate GC-induced actions, GCs can either be converted into inactive compounds (Wingfield, 2013), or a negative feedback pathway can be induced via GCs binding to receptors in the hypothalamus, hippocampus, and the pituitary, halting production rates and allowing

elevated levels to return to baseline (Nelson, 2005). Glucocorticoids support basic functions such as maintaining metabolism, locomotor activity, and reproductive events. Variation in GCs occurs between different species, sexes, and developmental life stages, as well as on a diel basis in individuals. GCs rise with increasing energetic demands such as reproduction, thermoregulation, and immune responses, as well as with environmental changes (Landys *et al*., 2006; Romero *et al.*, 2009; Hau *et al*., 2016).

When an organism experiences an acute stressor, circulating GC concentrations increase rapidly, often within 3 minutes and continue to rise up to 60 minutes after the onset of the stressor (Sapolsky *et al*., 2000; Romero, 2004; Landys *et al*., 2006). The rise in GCs aids in coping with stressors by mobilizing energy and increasing cardiovascular functions, locomotion, or foraging activity. While acute GC release can be beneficial to an organism in this way, prolonged or chronic GC release can have deleterious effects such as inhibition of reproductive and immune function, decreased cognition and brain cell numbers, and even mortality (Sapolsky *et al.,* 2000; Romero, 2004). Severe or prolonged stress can result in the dysregulation of the hypothalamic-pituitary-interrenal (HPI) axis, leading to permanently elevated or depressed levels of circulating GCs and a lack of ability to further moderate GC responses (McEwen & Wingfield, 2003). Without this ability, an organism can no longer mount a response to novel stressors and experiences defective metabolic, immune, and reproductive functions which can ultimately be fatal (Romero *et al.*, 2009).

Measuring GCs after exposure to a stressor is one way to assess the effectiveness of the HPI axis. This response can be measured by collecting “baseline” GC levels when an organism is acclimated to its environment with minimal stressors then exposing it to

short-term agitation, measuring its GC response and comparing it to the baseline (Glennemeier & Denver, 2002; Gabor *et al.*, 2013). If no differences are detected between these two measurements, then the organism either has a dysfunctional HPI axis or the stimuli was not stressful enough to elicit a response. While cortisol has successfully been used to measure responses to stressors (Hopkins *et al.*, 1997; Dickens & Romero, 2013; King *et al.*, 2016**;** Gabor *et al.*, 2018), it is also associated with other physiological processes and therefore should not be the only metric considered (Sapolsky *et al*., 2000; Le *et al.*, 2005; Dhabhar, 2009; Breuner *et al.*,2013). Plasma and serum glucose levels tend to rise when exposed to stressors in laboratory experiments (Ackerman *et al.*, 2000; Lankford *et al*., 2005; Costas *et al*., 2008), therefore glucose can be an additional indicator that an organism may be undergoing stress.

Natural history of *Gambusia affinis*

Gambusia affinis is a live-bearing freshwater fish found on every continent except Antarctica. The native range of *G. affinis* is primarily eastern North America including Texas, but this species has been introduced around the globe for their control of mosquito larvae (Lloyd *et al.*, 1986; Hubbs, 2000; Pyke 2005). Mosquitofish prefer water temperatures of 31–35℃ and calm, shallow water over turbulent water (Pyke, 2005), though they are found in a wide variety of environments including urban streams (Lloyd *et al.*, 1986; Page *et al.*, 2011).

Mature females grow to standard lengths (SL) of no more than 6 cm and an average mass of 0.2–1.0 g, and males reach SLs of 3.5 cm with an average mass of 0.13–0.2 g (Ryder, 1882; Hildebrand, 1919). Males are distinguished from females based on

the presence of a gonopodium, a modified anal fin used for passing sperm bundles to the urogenital opening of the female when mating (Constantz, 1989). The tip of the gonopodium is equipped with spines and barbs to hold it in place during its brief contact with the urogenital opening during the transfer of sperm (Rosen & Gordon, 1953; Pyke, 2005). Females often develop a dark peritoneal spot which is thought to be an indication of reproductive maturity (Stearns, 1983).

Courtship in *Gambusia* involves approaches by the male and ultimately acceptance or rejection by the female. Males tend to chase females while directing gonopodium thrusts at the female in attempt to copulate (Haynes, 1995). When successful, copulation takes an average of 0.9 seconds (Warburton *et al*., 1957). Fertilization occurs internally via the transfer of sperm bundles and young develop inside the female until they are born as free-swimming fish (Wourms, 1981). There is evidence of maternal provisioning (matrotrophy) in *Gambusia*, though they are generally considered to be lecithotrophic, or yolk dependent (Reznick & Miles, 1989; Marsh-matthews *et al*., 2001). Gestation is approximately 22–25 days, but varies with water temperature, season, and locality (Gall *et al*., 1980; Reznick, 1981).

Timing from birth to reproductive maturity is about 1–2 months (Pyke, 2005). Development includes three main phases: 1) the gestation phase, or the time of fertilization to parturition, 2) the immature phase where the fish transitions from immature to adult, and 3) the final stage where the fish are adult. *Gambusia* have a distinct breeding season from March to October (Pyke, 2005) and males and females show a near identical annual reproduction cycle (Self, 1940). Typically, it is several days to two weeks between birth of a brood and fertilization of the next, with intervals

between broods as low as 28–30 days (Turner, 1937; Reznick & Miles, 1989). Females can store viable sperm from one mating that can be used for several months and may produce multiple broods (Hildebrand, 1919; Krumholz, 1948); up to 9 broods in a season, although 4–5 is most common. Young females may only produce one or two broods in their first season (Krumholz, 1948; Maglio & Rosen, 1969). The average clutch size ranges from 5–100 eggs (Krumholz, 1948).

Gambusia are predatory and forage on a large variety of prey including insects, spiders, crustaceans, worms, mollusks, larvae and pupae of aquatic invertebrates, algae and other plant material, smaller fish (even of its own species), and diatoms (Odum & Caldwell, 1955; Crivelli & Boy, 1987). Often found foraging near the surface of the water, they rely primarily on sight to detect prey, predators, and objects (Lanzing & Wright, 1982). Foraging peaks in the morning and at dusk although they do forage during the day. *Gambusia affinis* are often preyed upon by piscivorous fish including largemouth bass (*Micropterus salmoides),* green sunfish *(Lepomis cyanellus*), warmouth (*Lepomis gulosus),* white crappie (*Pomoxis annularis*), longear sunfish (*Lepomis megalotis*), and bluegill (*Lepomis macrochirus*), along with snakes, birds, and invertebrates. Antipredator responses specific to *Gambusia* include morphological features such as large caudal regions, small heads, elongate bodies, and posterior ventral eye position. These adaptations allow for high-energy swimming bursts to evade capture (Langerhans *et al.*, 2004). *Gambusia* are also known to respond to conspecific chemical cues from skin extract (Garcia *et al.*, 1992). *Gambusia* are also known to form shoals in the presence of predators, a common antipredator response by fish (Pike, 2005).

Gambusia are widespread, tolerant, adaptable, and variable in their biology. Because *Gambusia* are widespread, abundant, and easy to maintain in a lab setting, they are an ideal organism to use for this study. Further, they are frequently found in urban water bodies. I examined how ALAN affects physiology, behavior, and reproduction of female *G. affinis* in the laboratory and in an outdoor mesocosm environment. These findings provide insight to the impacts of ALAN on this species and indicate that less tolerant fish will be more negatively affected.

II. ARTIFICAL LIGHT AT NIGHT ALTERS PHYSIOLOGY, BEHAVIOR, AND REPRODUCTION IN WESTERN MOSQUITOFISH (*GAMBUSIA AFFINIS*)

Introduction

Anthropogenic disturbances contribute to habitat loss and alteration, climate change, and exploitation of natural resources (Ellis, 2011; Dudgeon *et al.*, 2006; Vörösmarty *et al.*, 2010; Helm *et al.*, 2013), and are associated with shifts in water quality, water flow, seasonal timing, sound, and light pollution (Jenkins, 2003; Allan, 2004; Barbier, 2012; Davies *et al.*, 2014; Swaddle *et al.*, 2015; Shannon *et al.*, 2016; Buxton *et al.*, 2017; Sordello *et al.*, 2019). Artificial light at night (ALAN), artificial light that alters the natural light and dark cycle in an ecosystem, is one type of anthropogenic pollutant (Swaddle *et al.*, 2015). Light plays a key role in the ecology of organisms as a source of energy and information, a regulator of circadian rhythms, and as a cue for communication, navigation, and orientation (Gaston *et al.*, 2012, 2017). As worldwide population size and urbanization increases, ALAN has become so widespread that 83% of the global human population lives under light-polluted skies, and 40% lives in areas that are continually illuminated due to ALAN (Cinzano *et al*., 2001; Swaddle *et al.*, 2015; Falchi *et al.*, 2016). Hölker *et al.*, (2010) summarized data showing that ALAN is increasing alongside urbanization at an average rate of 6% per year (range 0-20%). Despite this increase in ALAN and the known consequences on ecosystem health and biodiversity, there are still gaps in our understanding of how ALAN affects organisms' physiology and behavior; especially those organisms that occupy aquatic habitats.

Global freshwater systems contain 9.5% of all species, and these freshwater species are declining at rates exceeding those of tropical rainforests, primarily due to anthropogenic stressors (Ricciardi & Rasmussen, 1999; Xenopoulos *et al*., 2005; Dudgeon *et al*., 2006; Balian *et al*., 2008). Moreover, 90% of the human population lives within 10 km of a body of water and 50% within 3 km, making freshwater and coastal marine areas the most impacted by anthropogenic disturbances, such as ALAN (Dudgeon *et al.*, 2006; Kummu *et al.*, 2011; Venohr *et al.*, 2018). Aquatic organisms are affected by ALAN because they are influenced by photoperiod across life history stages, including reproduction, growth, development, and activity (Downing & Litvak, 2002; Mehner, 2012). ALAN has detrimental effects on behavior, reproduction, foraging, orientation, predation, physiology, and migration in various taxa (Longcore & Rich, 2004; Navara & Nelson, 2007; Hölker *et al.*, 2010; Ouyang *et al.*, 2011; Gaston *et al.*, 2013; Davies *et al.*, 2014; Gaston *et al.*, 2014, 2015), yet, most studies of ALAN focus on terrestrial taxa. Of the studies of ALAN on aquatic organisms, many are on marine systems, leaving a knowledge gap regarding how ALAN affects freshwater organisms (Depledge *et al.*, 2010; Perkin *et al.*, 2011).

Organismal response to stressors, such as ALAN, can be quantified by measuring the amount of cortisol, the primary glucocorticoid (GC) in fishes (Idler & Truscott, 1972), that is released in response to a potential stressor (Hopkins *et al.*, 1997; Dickens & Romero, 2013; King *et al.*, 2016**;** Gabor *et al.*, 2018). When a fish encounters a stressful event, its hypothalamo-pituitary-interrenal (HPI) axis is activated, releasing GCs into the bloodstream, mobilizing energy, thus allowing the organism to alter behavior and physiology and maintain homeostasis (Wendelaar Bonga, 1997; Romero, 2004). When

faced with an acute stressor, this mechanism is adaptive, but prolonged exposure to a stressor can have harmful, long-term, and even fatal effects (Sapolsky *et al.*, 2000; Romero, 2004). Elevated GCs are linked to lower survival, reproduction, and dysregulation of immune responses (Bonier *et al.*, 2009) but can also enhance immunity, growth, and reproductive output (Dhabhar *et al.,* 1995; Dhabhar & McEwen, 1996; Dhabhar & Viswanathan, 2005; Viswanathan *et al.*, 2005; Thawley & Kolbe, 2020). Because GCs are also involved in altering other physiological processes (Sapolsky *et al*., 2000; Le *et al.*, 2005; Dhabhar, 2009), if ALAN is a stressor that causes changes to cortisol release rates, it can indirectly affect downstream traits—such as behavior, growth, and reproduction. ALAN can have direct and indirect effects on organisms. It can directly alter their behavior as evidenced in studies where fish increase their activity, alter shoaling behavior, and spend more time in open (riskier) areas under ALAN (Becker *et al.*, 2013; Foster *et al.,* 2016; Kurvers *et al.*, 2018; Sanders & Gaston 2018; Czarnecka *et al.*, 2019). There are also many indirect effects of ALAN on taxa. ALAN results in increased blood glucose in goldfish, *Carassius aurarus*, (Ryu *et al.* 2019), impaired melatonin rhythms in European perch, *Perca fluviatilis*, and reduced hatching success in clownfish, *Amphiprion ocellaris,* (Fobert *et al.,* 2019). Direct and indirect effects on ALAN on organisms should be measured to fully understand its consequences.

Here, I propose to quantify how ALAN affects multiple traits of western mosquitofish (*Gambusia affinis*). These are small, livebearing fish native to eastern North America, but introduced globally, and are found in a wide variety of environments, including urban streams (Lloyd *et al.,* 1986; Hubbs, 2000; Pyke 2005; Page *et al*., 2011). This species is generally found in shallow waters and forages near the surface of the

water primarily at morning and at dusk, though sometimes during the day (Hess & Tarzwell, 1942; Belk & Lydeard, 1994). This species is considered tolerant due their success as invaders and their ability to live in adverse conditions (Cherry *et al.*, 1976; Lloyd *et al.*, 1986; Pyke, 2008). I used laboratory and mesocosm experimental approaches to test the hypotheses that ALAN alters the physiology, behavior, and reproduction of *G. affinis*.

Materials and methods

Experiment 1: Laboratory exposure of female mosquitofish to ALAN: consequences on physiological stress, behavior, and fitness correlates

Reproduction and cortisol

I collected *Gambusia affinis* from the Blanco River in Hays County, Texas during part of the breeding season (13 April 2018 and 13 June 2018) with a seine and transported them to the laboratory. The collection site was exposed to 0–0.1 lux of illumination. I took lux measurements using a digital lux meter (Dr. Meter, model LX1330B). I placed fish in 38 L tanks and fed them ISO flake food (TetraMin) daily and supplemented 3 times a week with brine shrimp. One week after collection, I haphazardly placed 80 individual mature females each into semi-clear containers that had small holes in the bottom and that were located above another container. The holes allowed any offspring that were born to escape into the lower container and avoid maternal cannibalism (following Cazan and Klerks 2015). Each of the containers also had small holes on the sides to facilitate flow of chemical cues of conspecifics to mimic shoaling and simultaneously allow for individual sampling. Eight of the containers were placed

together in a 38 L tank filled with 28.5 L of conditioned tap water and gravel across the bottom (Fig. 1).

On 21 April 2018 I exposed 5 of these tanks (each with the 8 smaller containers; N = 40 females), to a control treatment of 14:10 h light: dark cycle and 5 other tanks (N = 40 females) to the experimental treatment (ALAN) of 24:0 h light: dark cycle for 60 days. On 23 June 2018 and 5 September 2018, I repeated the experiment with the same set-up for another 59 and 50 days respectively, for a total of 15 replicates per treatment. I simulated daylight with a full spectrum fluorescent light (MingDak) at 880 lux and ALAN using LED lights (Utilitech) at 120 lux. Full daylight levels can reach up to 25,000 lux with illuminances up to 100,000 lux in direct sunlight (Blume *et al.*, 2019). Light levels at night in Hays County, Texas near the collection sight ranged from 16 lux at dim streetlamps to 230 lux at flood lights, therefore this nighttime lux level is ecologically relevant. I hung all lights 51 cm above the tanks. After the first round (60 days), I increased the daylight to 2,380 lux and ALAN to 246 lux because it was unclear that the lower levels were sufficiently high for the fish to respond.

I monitored the containers daily for offspring. Females generally drop all offspring in a brood at once, therefore when present, I recorded the date, number of offspring, and condition of offspring in each brood. Offspring condition was defined as 1) normal: offspring were alive and appeared healthy or 2) dead: offspring were deceased. Fish were fed ISO flake food (TetraMin) daily. I changed the tank water by siphoning out ¾ of the water from the bottom (to remove feces) and replacing it with conditioned tap water every two weeks.

During the first two rounds, I collected water-borne hormones two days after the females had been placed in their experimental container (but light exposure had not yet begun) and thus had an opportunity to acclimate to the experimental set-up (day 0), then again on day 7, 30 and the last day of the round. After the last "baseline" hormone sample was taken on the last day (60, 59, 50) I assessed whether the fish were chronically stressed (as indicated by responsiveness of the HPI axis) by agitating the fish while collecting their hormones. The methods I used for hormone collection followed Blake *et al*., (2014). I placed each fish in a LDPE plastic insert in a 250 ml glass beaker with 60 ml of dechlorinated water for 30 mins after which time I recorded mass (g) and standard length (mm) then returned the fish to its original container. For the agitation test, I placed fish into the same set-up as above and shook them for one minute every other minute for a total of 30 mins. Each hormone collection event began at 0900 hours to control for natural diel fluctuations of cortisol. I cleaned beakers and inserts with 95% ethanol and rinsed them with DI water before use and handled them with non-powdered gloves to prevent contamination. Scott *et al*., (2008) tested this non-invasive method for establishing cortisol release rates from fish and Blake *et al*., (2014) validated this method of analyzing cortisol from water-borne hormones using *Gambusia geiseri*, a close relative of *G. affinis.*

Shoaling behavior

I removed fish from individual containers after day 60 in the ALAN experiment and placed them into plastic containers (33.02 cm x 20.32 cm x 11.43 cm) in groups of 4, a shoal size used in previous studies with Poeciliidae (Tobler & Schlupp, 2008). I filled containers (lined on the outside with white paper) with 5 L of conditioned tap water.

Groups acclimated in the containers for 30 mins and I then recorded the groups under their respective treatment for ~24 hours with webcams using ManyCam software. I analyzed videos using EthoVision XT (Noldus) for total activity and proximity to each other during day (14 h) and night hours (10 h).

Individual and hiding behavior

Using fish from the second round of exposure for this experiment, I set up 18 L tanks with one clear half cylinder of PVC and one white half cylinder of PVC which were randomly assigned to each side of the tank. I filled the tanks with 1.3 L of treated tap water (just enough to cover the arches). I covered the sides of the tanks with black paper to prevent the fish from being distracted from their surroundings. At night, I removed fish from the ALAN treatment (after day 50) and individually placed them into the testing tank and allowed the fish to acclimate under a clear, plastic 1 L container for 10 min. Following acclimation, I removed the clear container and began recording activity with ManyCam software for 10 min. I analyzed videos using EthoVision XT (Noldus) for: (1) time spent "hiding" under either arch, (2) time spent moving, (3) velocity, and (4) acceleration of movement.

Statistical analysis

I used a repeated measure generalized linear mixed model (rm GLMM) with natural log transformed cortisol release rates with treatment and day as the fixed effects and ID as the random effect to account for repeated measures. When there were significant fixed effects differences, I used *post hoc* Tukey's HSD comparisons. To

explore effects of ALAN on reproduction (number and condition of offspring) I used a chi-squared test. I used a rm GLMM to analyze changes in mass and standard length over time with treatment and time as fixed effects and individual as the random effect. To determine the effects of ALAN on shoaling behavior, I used a MANOVA. For all other behavioral analyses, I used generalized linear models with SL and treatment as model effects. I used JMP Pro 14.0.0 (SAS Institute, Inc.) for all statistical analyses.

Experiment 2: Mesocosm exposure of female mosquitofish to ALAN: consequences on physiological stress and behavior

Reproduction, cortisol, and glucose

I constructed 16 mesocosms using 62.45 L clear, #5 (polypropylene) plastic containers with six 5 cm holes drilled in the sides and covered with mesh for water drainage while preventing fish from escaping (Fig. 2). I cut out the center plastic of the lids and replaced it with mesh to allow light to pass through. After construction I placed mesocosms outdoors underneath 60% shade cloth. On 25 June 2019, I added 48 L of water to each mesocosm. The following day I added 4 L of sediment collected from the Blanco River in Hays County, Texas and 1 L of pond water. I added 1 L aliquots of zooplankton to each mesocosm on 29 June 2019 and 2 July 2019. I added 16 pieces of ceramic bio media (11 BrightWater Bio Media and 5 Fluval BioMax) to each mesocosm to provide a surface for bacteria to grow. Additionally, I added one sponge filter to each mesocosm to prevent the buildup of ammonia and nitrates. I added two fake breeder plants (Penn Plax Aquarium Breeding Grass) to each mesocosm for habitat cover and to provide refuge for potential offspring.

From 19 June–21 June 2019 I collected 240 *G. affinis* from the Blanco River in Hays County, Texas using dipnets and seines, transported them to the lab, and fed them ISO flake food (TetraMin) daily. On 27 June 2019 I marked 64 females with white, red, orange, or yellow elastomer tags (N = 16 per color). On 3 July 2019 I haphazardly placed five tagged females, eight non-tagged females, and two males (totaling 15 fish) into each mesocosm. I hung two lights (Onforu 35 W LED Flood Lights; 3300 lumens 5000 K) 52 cm above half of the mesocosms. Five days after placing fish into mesocosms, I turned on the artificial night lights on the experimental (ALAN) side (day 0) exposing 8 mesocosms (N = 120 fish) to 24 hours of light. These lights were on from 2000–0600 h and ranged from 260–280 lux at the top of the mesocosms and 155–175 lux at the surface of the water. I conducted lux measurements using a digital lux meter (Dr. Meter, model LX1330B). To prevent light from reaching the control mesocosms at night I hung a black plastic curtain in between treatment blocks.

I collected "baseline" cortisol from each tagged female on day 4 to capture the potential effect of treatment while giving enough time to acclimate to the setup. Beginning at 0900 hours I placed each fish in a LDPE plastic insert in a 250 ml glass beaker with 60 ml of spring water for 30 mins. After 30 mins I collected the water sample then measured and recorded the mass (g) and standard length (SL; mm), then returned the fish into its original mesocosm. Using the same methods, I collected baseline cortisol from the same tagged individuals on days 16 and 32. Immediately after baseline cortisol collection on day 32, I collected agitation cortisol release rates to test for a stress response (following the lab protocol above). Before each cortisol collection I cleaned beakers and inserts with 95% ethanol and rinsed them with DI water. During collection I wore non-

powdered gloves. On day 32 I euthanized fish in an ice slurry for 30 mins then dissected 5–6 non-tagged females per mesocosm to obtain the brain, muscle, and liver tissues. I placed like tissues from each mesocosm into 1.5 ml Eppendorf tubes filled with ExCell PLUS fixative for 24 hours then moved to new tubes of 70% ethanol for later analysis.

I recorded water quality parameters (ammonia, nitrites, nitrates, total dissolved solids, temperature, conductivity, pH, and salinity) from each mesocosm twice a week. I added dechlorinated tap water to the mesocosms when it was lower than the drainage holes.

Individual and hiding behavior

I repeated the hiding behavioral experiment following the methods from experiment 1 (above) using tagged females from each mesocosm but performed the experiment during the day (instead of at night) and with a 5 min acclimation period.

Statistical analyses

To examine effects of ALAN on cortisol release rates, I performed a repeated measure generalized linear mixed model (rm GLMM) with natural log-transformed cortisol standardized by standard length (SL) with treatment as the fixed effect and individual nested in mesocosm as the random effect. When there were significant treatment effects, I used *post hoc* Tukey's HSD comparisons. To assess effects of ALAN on *G. affinis* glucose levels, I used a GLMM with treatment as the main effect and mesocosm as the random effect. I used a rm GLMM to analyze changes in mass and standard length over time with treatment and day as fixed effects and individual nested in mesocosm as the random effect. For behavior analyses, I used generalized linear mixed

models with SL and treatment as fixed effects and individual nested in mesocosm as the random effect. To explore differences in survival I ran a Log-Rank survival analysis. I used JMP Pro 14.0.0 (SAS Institute, Inc.) for all analyses.

*Cortisol extraction, reconstitution, and enzyme immunoassays (EIA***)**

I stored water-borne hormone samples at –20 °C until thawed for extractions following methods of Gabor *et al.* (2016). I pulled water samples through Tygon tubing under a vacuum into C18 solid phase extraction (SPE) columns (SepPak Vac3 cc/500 mg; Waters, Inc., Milford, MA, USA). SPE columns were primed with 4 ml of 100% HPLC grade methanol followed by 4 ml of distilled water. I then eluted columns with 4 ml 100% HPLC grade methanol into borosilicate vials then evaporated the samples by placing them in a 37 °C water bath while under nitrogen gas. I resuspended the residue in 5% ethanol (95% lab grade) and 95% EIA buffer (Cayman Chemical, Inc) to a total volume of 720 µl then diluted samples to 1:16 before plating.

I measured cortisol release rates in duplicate for all samples using EIA kits (No. 500360, Cayman Chemical Company, Inc.). The cortisol assays have a range from 6.6 to 400 pg/ml and a sensitivity of approximately 35 pg/ml. Sample absorbances were read on a spectrophotometer plate reader at 405 nm (BioTek 800XS). I multiplied cortisol release rates (pg/ml) by the final resuspension volume (0.720 ml), divided by the SL (mm) of the individual, and multiplied that value by 2 (because collection was only a half hour) for a final unit of pg/mm/hr. Inter-plate variation was 12.35% for the laboratory experiment (5 plates) while intra-plate variation ranged from 0.39% to 14.88%. For the mesocosm experiment (6 plates), inter-plate variation was 11.53% and intra-plate variation ranged from 0.45% to 6.95%.

Glucose extraction, reconstitution, and colorimetric assays

I stored tissue samples at –20 °C until thawed for extractions. For each mesocosm, I combined like tissues from 6 fish and weighed the tissues before adding them to 1.5 ml Eppendorf tubes with 400 µl of 100% ethyl alcohol. After homogenization in the tubes, I centrifuged samples for 10 minutes at 10,000 rpm at 4°C. I removed the supernatant of each sample and transferred it to a new Eppendorf tube which was then placed in a vacufuge overnight. I reconstituted the samples using 100 µl of 1 M phosphate buffer saline (PBS).

I measured glucose levels in duplicate for all samples using Eppendorf Cell Culture 96-well plates. Samples were diluted to ½ by adding 25 µl of 1 M PBS per 25 µl of sample. Twenty-five µl of 10 mM sodium acetate trihydrate (pH 5) and 10 µl of 150 mM PBS was added to all wells. I mixed plates then incubated them for 1 hour at 40°C. After incubation, 25 µl of 150 mM PBS (pH 7.4) was added to each well, followed by 25 µl of enzyme mix. The enzyme mix contained ampliflu red stock solution, horseradish peroxidase, glucose oxidase working solution, 150 mM sodium phosphate buffer, and water. This combination results in the oxidation of ampliflu red which causes a color change to a pinkish resorufin in the microplate well. Resorufin has an absorbance of 560 nm which is proportional to the concentration of glucose. After the enzyme mix was added, I incubated the plates at room temperature for 30 minutes and read the absorbances on a spectrophotometer plate reader at 570 nm (accuSkan FC) every 5 minutes. Inter-plate variation was 10.22% and intra-plate variation ranged from 6.93% to 12.19% (3 plates).

Results

Reproduction, cortisol, and glucose

Offspring number ($\chi^2 = 16.272$, $p = 0.434$) and condition ($\chi^2 = 3.073$, $p = 0.081$; Fig. 3) did not differ between treatments.

In the laboratory experiment, I found no significant difference in cortisol release rates between fish in the control vs ALAN treatment (rm GLMM: treatment x day: $F_{4,155} = 1.439$, $p = 0.224$; Table 1; Fig. 4a). Fish had significantly higher agitation cortisol release rates compared to baseline after 60 days irrespective of treatment (day: $F_{4,155} = 103.316$, $p < 0.0001$; Fig. 3a), indicating the fish from both treatments could mount a stress response.

In the laboratory experiment, I found no significant effect of ALAN on mass (rm GLMM: treatment x time: $F_{3,460} = 0.302$, $p = 0.824$) or SL ($F_{3,460} = 0.253$, $p = 0.860$) over the duration of the experiment.

In the mesocosm experiment, I found no significant differences in cortisol release rates between treatments across days (rm GLMM: treatment x day: $F_{3,138} = 2.099$, $p = 0.103$; Table 2; Fig. 4b). Similar to the lab study, cortisol release rates were higher after agitation compared to baseline for both treatments (day: $F_{3,138} = 4.998$, $p = 0.003$; Fig. 3b), indicating the fish could mount a stress response.

In the mesoscosm experiment, I found significantly higher glucose levels in the brain tissues of fish from the control treatment than fish from the ALAN treatment (GLMM: treatment: $F_{1,13} = 8.039$, $p = 0.014$; Fig. 5). There was no effect of treatment on any other tissues (all $p > 0.05$; Table 3).

In the mesocosm experiment I found no significant effect of ALAN on SL (rm GLMM: treatment x day: $F_{3,138} = 0.953$, $p = 0.417$) or mass ($F_{3,138} = 0.953$, $p = 0.417$), however, fish in both treatments increased in SL (day: $F_{3,138} = 16.171$, $p < 0.0001$) and lost mass ($F_{3,138} = 78.258$, $p < 0.0001$) over the duration of the experiment. There were no differences in survival between treatments (Log-Rank Survival Analysis: $\chi^2 = 0.887$, $p = 0.346$).

Ammonia and nitrites were not detected at any point in the mesocosms. Total dissolved solids, temperature, conductivity, pH, and salinity did not differ among mesocosms ($p > 0.05$).

Shoaling behavior

After being in the laboratory experiment for 59 days, female *G. affinis* in the ALAN treatment spent significantly less time shoaling during the day than the control treatment (MANOVA: time x treatment: F = 5.917, $p = 0.256$; Fig. 6). Fish from both treatments moved a significantly greater distance (time: F = 8.051, $p = 0.011$), had a significantly higher velocity (F = 7.822, $p = 0.012$) and greater acceleration (F = 5.930, $p = 0.026$) during the day than at night. Time not moving was significantly positively correlated with baseline cortisol (Kendall's τ: $\tau = 0.571$, $p = 0.048$).

Individual and hiding behavior

In the laboratory individual and hiding experiment, after exposure to ALAN, I found that fish spent significantly more time not moving (LMM: treatment: $F_{1,1} = 6.929$, $p = 0.015$; Table 4; Fig. 7a) and had significantly lower velocity ($F_{1,1} = 10.099$, $p =$

0.005; Fig. 7b) than fish in the control treatment. I found no significant effect of ALAN on time spent hiding at night ($F_{1,1} = 0.018$, $p = 0.896$).

In the mesocosm experiment I found no significant differences in time not moving, velocity, or time spent hiding (all $p > 0.05$; Table 5) between treatments. There were no significant correlations between any activity parameter and cortisol (Kendall's τ: $p > 0.05$).

Discussion

With the spread of urbanization, light pollution will increase world-wide (Hölker *et al.*, 2010; Davies *et al.*, 2014; Falchi *et al.*, 2016) and has the demonstrated potential to negatively impact exposed organisms in direct and indirect ways by affecting their physiology, behavior, and reproduction. I found that the western mosquitofish, *G. affinis,* a widespread, and in many locales, invasive species, responds to ALAN by decreasing activity (both individually and around conspecifics) and exhibiting reduced brain glucose levels compared to those exposed to normal light dark cycles. I did not, however, observe a change in cortisol release rates, growth, survival, or reproduction in response to ALAN. These results suggest that *G. affinis* may behaviorally adjust to the perturbation of ALAN rather than respond via a change in the regulation of the HPI axis. The lack of a cortisol response by *G. affinis* to ALAN may play a role in their success as invaders and establishing populations in water with varying physical properties (Lloyd *et al.*, 1986; Hubbs, 2000).

When fish were exposed to an agitation treatment, cortisol release rates were significantly higher than baseline levels for fish from both ALAN and control groups,

indicating that they were capable of mounting a stress response. Therefore, the lack of a significant effect of ALAN on cortisol release rates under laboratory conditions and in mesocosms was not due to a dysregulated HPI axis, meaning they were not chronically stressed. In the laboratory experiment, I also measured cortisol release rates on day 2 and found that they were significantly elevated in both treatments as compared to days 0 and 7, suggesting that fish were not acclimated to the experimental setup. By day 7, cortisol levels returned to baseline in both treatments. In the mesocosm study, I tested the hypothesis that there was a transient cortisol response to ALAN sometime between days 2 and 7 by collecting cortisol on day 4; however, I still did not detect a change in cortisol in response to ALAN. My results agree with other studies which found a lack of glucocorticoid response to ALAN using lux levels comparable or greater (15–300 lux) to ours (155–246 lux). For example, Szekeres *et al.* (2017) found that juvenile bonefish (*Albula vulpes*) exhibit increased blood glucose in response to ALAN but there was no effect of ALAN on whole body cortisol. Additionally, European perch (*Perca fluviatilis*) exposed to ALAN had decreased melatonin production compared to the control group, but there were no differences in cortisol between treatments (Brüning *et al.*, 2015). There were no differences in corticosterone levels between control and ALAN-exposed treatments in brown anoles (*Anolis sagre;* Thawley & Kolbe, 2020) and wood frogs (*Lithobates sylvaticus*; May *et al.*, 2019). These results suggest there are other mechanisms of coping with stressors (Ouyang *et al.*, 2018) or ALAN may simply not elicit a hormonal response in many species (Grunst *et al.*, 2019), including *G. affinis*. The species tested thus far may be more tolerant species as they are abundant, so testing less tolerant species be necessary to fully understand the implications of exposure to ALAN.

Female *G. affinis* from mesocosms exposed to ALAN had higher glucose levels in their brain tissue compared to fish kept under control light conditions. Goldfish (*Carassius auratus*) and bonefish (*Albula vulpes*) had elevated plasma or blood glucose levels after exposure to ALAN (Szekeres *et al.,* 2017; Ryu *et al.,* 2019), however, the increase of glucose in blood or plasma is likely due to a decrease in tissue usage (Horner *et al.*, 1987; MacDougall-Shackleton *et al.,* 2019). An increase in brain glucose indicates that *G. affinis* decreased usage of their brains in the ALAN treatment by reallocating glucose to from brain tissue to elsewhere in the body. Experiments on the cognitive ability of fish could elucidate whether or not there are differences in brain function between fish exposed to ALAN and those kept under a natural light cycle.

The activity of *G. affinis* was overall reduced both individually at night, and in shoaling during the day after exposure to ALAN. Fish moved slower and less often at night after 50 days of exposure to ALAN in the laboratory. Because they did not spend more time hiding at night, this could put them at greater risk of predation. This result opposes several other studies which found an increase in activity in fish (Becker *et al.,* 2013; Foster *et al.,* 2016; Kurvers *et al.*, 2018; Czarnecka *et al.*, 2019), American toads (*Anaxyrus americanus*), zebra finches (*Taeniopygia guttata*), and anoles (*Anolis leachii* and *A. wattsi* (Dananay & Bernard, 2018; Batra *et al.*, 2019; Maurer *et al.*, 2019) after exposure to ALAN. May *et al.* (2019), however, found that *Lithobates sylvaticus* tadpoles were also less active in the day and night after exposure to ALAN and Buchanan *et al.* (1993) found that grey treefrogs, *Hyla chrysoscelis,* reduced foraging activity at night under ALAN. These results suggest that the effects of ALAN vary by species. Female *G. affinis* also spent less time shoaling during the day after the same exposure to ALAN in

the laboratory. Shoaling is beneficial as a defense against predation and generally results in more efficient foraging, therefore a lack of shoaling during the day could leave them more susceptible to predation and affect their ability to find food (Pitcher, 1986; Laland & Williams, 1997). In the control group, there was a clear display of diel shoaling activity where fish were more active and swam in closer proximity during the day than at night. This diel activity pattern disappeared after exposure to ALAN. These results align with studies of fish where shoaling was decreased after exposure to other disturbances such as psychotropic drugs and parasites (Tobler & Schlupp, 2008; Green *et al.*, 2012). Overall, in the laboratory, *G. affinis* exposed to ALAN moved slower and less often at night and also shoaled less during the day, which could leave them more susceptible to predation at all times.

Offspring number and condition did not differ significantly between treatments. Most females did not experience parturition during the experiment and consequently my sample size was low. Offspring counts could have been affected by cannibalism as well as this species' ability to resorb embryos under suboptimal conditions (Meffe & Vrijenhoek, 1981). Offspring number and condition was not measured in mesocosms because there were no offspring observed in either treatment. In previous studies, continuous light cycles resulted in earlier hatching and smaller larvae size of haddock, *Melanogrammus aeglefinus*, embryos, complete failure of embryo hatching in the common clownfish, *Amphiprion ocellaris*, under a much lower lux level (26 lux), and early stage pregnancy termination in female white rats, *Rattus norvegicus* (Downing & Litvak, 2002; Berbets *et al.*, 2019; Fobert *et al.*, 2019).

As discussed above, pervasive effects of ALAN have previously been reported and here I show additional adverse effects on a tolerant, invasive species. Since urbanization is on the rise, it is important that mitigation efforts take place to minimize these impacts (Falchi *et al.,* 2011). In areas where such efforts have already taken place, night light is successfully conserved without compromising human safety or security (Kyba *et al.,* 2015; Steinbach *et al.,* 2015; Kyba *et al.,* 2017). Additionally, few experiments have examined how various light color effects different taxa, which could potentially become a mitigation strategy. For example, Ryu *et al.,* (2019) found that dimming light induces less stress in goldfish, *Carassius auratusthan*, than sudden light changes. Expansion on this type of research needs to be conducted to combat the negative impacts of increasing light pollution. ALAN did not compromise every variable I measured; however, the reduction of activity and brain glucose in *G. affinis* are enough to decreases chances of survival. Therefore, measuring multiple metrics is necessary to fully comprehend the impacts of ALAN on organisms. Furthermore, reducing ALAN, including that near aquatic habitats, may aid in diminishing the negative effects on biodiversity.

Tables

Table 1 Results from a generalized linear mixed model testing the effects of ALAN on cortisol release rates of female *G. affinis* in the laboratory. 'Treatment' and 'day' were included as fixed effects and 'ID' was included as the random effect. Statistically significant values are in bold.

	Estimate ± SE	*t value*	*p* - value
Intercept	3.678±0.060	61.39	**<0.0001**
Control	0.038±0.060	0.63	0.5298
Day 7	-0.632±0.104	-6.10	**<0.0001**
Day 30	-0.863±0.104	-8.34	**<0.0001**
Day 60	-0.529±0.104	-5.11	**<0.0001**
Control*Day 7	-0.145±0.104	-1.41	0.1630
Control*Day 30	-0.145±0.104	-1.40	0.1642
Control*Day 60	0.130±0.104	1.26	0.2123

Table 2 Results from a generalized linear mixed model testing the effects of ALAN on cortisol release rates of female *G. affinis* in mesocosms. 'Treatment' and 'day' were included as fixed effects and 'ID' nested in 'mesocosm' was included as the random effect. Statistically significant values are in bold.

	Estimate ± SE	*t value*	*p* - value
Intercept	4.225±0.040	105.60	**<0.001**
Control	0.046±0.040	1.14	0.2582
Day 4	0.013±0.056	0.24	0.8101
Day 16	-0.146±0.056	-2.60	**0.0104**
Day 32	-0.062±0.056	-1.10	0.2725
Control*Day 4	0.131±0.056	2.34	**0.0205**
Control*Day 16	-0.084±0.056	-1.50	0.1346
Control*Day 32	-0.002±0.056	-0.04	0.9692

Table 3 Results from a generalized linear mixed model testing the effects of ALAN on glucose levels of female *G. affinis* tissues in mesocosms. 'Treatment' was included as the fixed effect and 'mesocosm' was included as the random effect. Statistically significant values are in bold.

Tissue		Estimate ± SE	*t value*	*p* - value
Muscle	Intercept	0.222±0.022	10.23	**<0.0001**
	Control	0.021±0.022	0.95	0.3588
Liver	Intercept	7.013±0.812	8.64	**<0.0001**
	Control	-0.317±0.812	-0.39	0.7020
Brain	Intercept	3.645±0.274	13.29	**<0.0001**
	Control	0.780±0.274	2.84	**0.0140**

Table 4 Results from a generalized linear model testing the effects of ALAN on behavior of female *G. affinis* in the laboratory. 'Treatment' and 'SL' were included as the fixed effects. Statistically significant values are in bold.

		Estimate ± SE	T value	p-value
velocity	Intercept	0.920±1.308	0.70	0.4901
	SL (mm)	0.038±0.038	1.01	0.3250
	Control	0.492±0.155	3.18	**0.0047**
	SL*Control	0.025±0.038	0.67	0.5134
Not moving	Intercept	246.189±167.558	1.47	0.1559
	SL (mm)	0.031±4.835	0.01	0.9949
	Control	-52.167±19.818	-2.63	**0.0152**
	SL*Control	-4.683±4.835	-0.97	0.3433
Hiding	Intercept	-50.340±140.142	-0.36	0.7229
	SL (mm)	2.864±4.044	0.71	0.4862
	Control	2.193±16.576	0.13	0.8960
	SL*Control	-0.395±4.044	-0.10	0.9232

Table 5 Results from a generalized linear mixed model testing the effects of ALAN on behavior of female *G. affinis* in mesocosms. 'Treatment' and 'SL' were included as the fixed effect and 'ID' nested in 'mesocosm' was included as the random effect. Statistically significant values are in bold.

		Estimate ± SE	T value	p-value
velocity	Intercept	7.116±4.295	1.66	0.1058
	SL (mm)	0.010±0.137	0.07	0.9443
	Control	-0.301±0.597	-0.50	0.6173
	SL*Control	-0.010±0.137	-0.07	0.9430
Not moving	Intercept	-0.066±80.778	-0.00	0.9994
	SL (mm)	4.609±2.576	1.79	0.0816
	Control	-2.760±11.234	-0.25	0.8072
	SL*Control	-2.506±2.576	-0.97	0.3368
Hiding	Intercept	28.867±94.445	0.31	0.7615
	SL (mm)	1.704±3.012	0.57	0.5748
	Control	3.166±13.135	0.24	0.8108
	SL*Control	-2.012±3.012	-0.67	0.5081

Figures

Fig. 1 Experimental setup for ALAN and control groups in the laboratory. Each container had 1 female *Gambusia affinis* in the top portion and a bottom container for offspring to potentially escape cannibalism (colored section). The container on the right shows the small holes used to allow chemical cues to disperse in the tank so that females could see and smell conspecifics.

Fig. 2 Mesocosm set up. Each container had six holes in the side and a lid covered with mesh and was filled with dechlorinated water, aliquots of sediment, pond water, zooplankton, two artificial breeder plants, ceramic bio media, and a sponge filter.

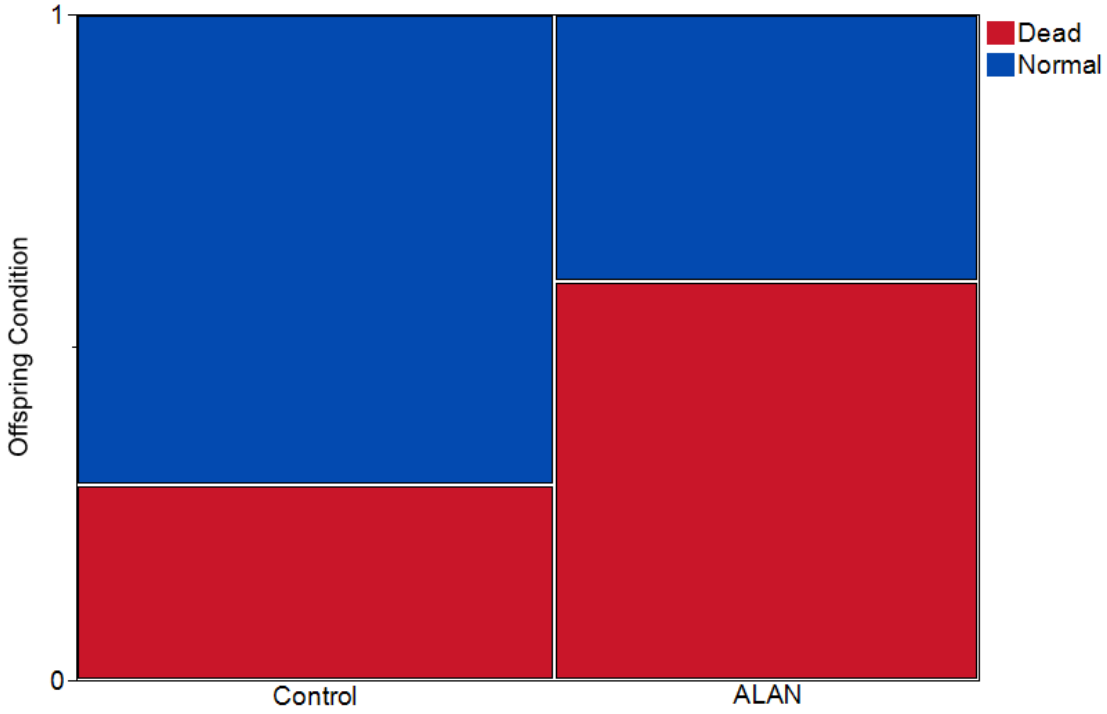

Fig. 3 The effects of ALAN on offspring condition of broods from female *G. affinis* in the laboratory (N = 32).

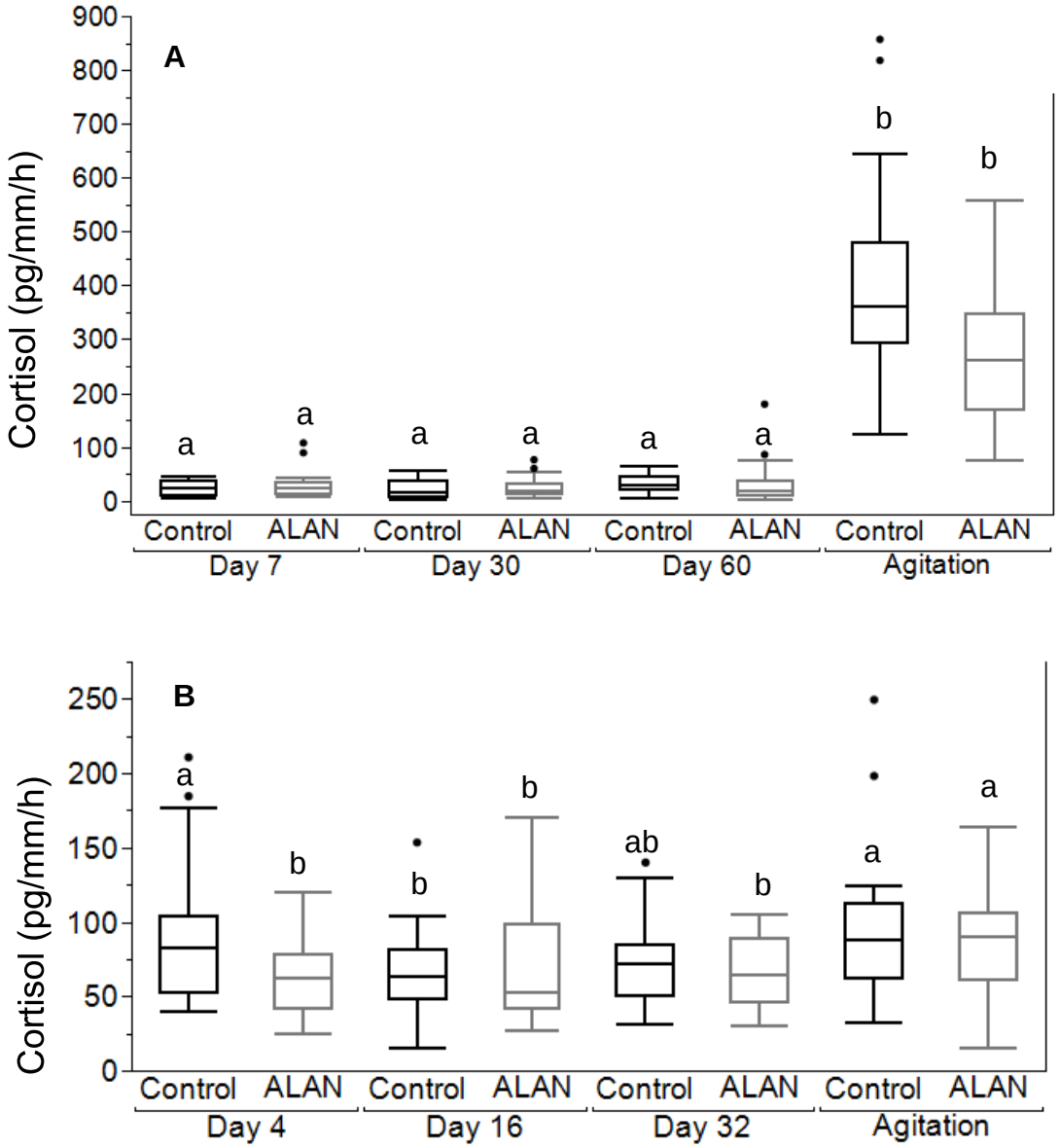

Fig. 4 Cortisol release rates (pg/mm/h) obtained from female *G. affinis* after 30 min of baseline and agitation collection in the (A) laboratory and (B) mesocosm experiment. Agitation was obtained on day 60 in the lab and day 32 in mesocosms. One high agitation value was excluded from the laboratory ALAN group to increase the spread of the figure. Box plots indicate median, range and first and third quartiles. Dots indicate outliers. Different lowercase letters indicate significant differences among treatment groups from Tukey's HSD comparisons**.**

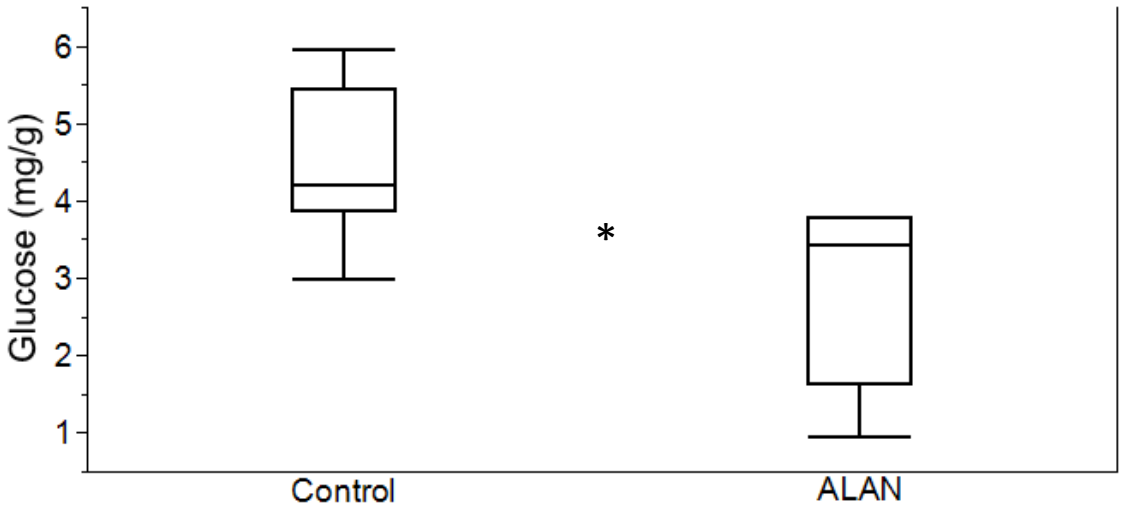

Fig. 5 Glucose levels (mg/g) obtained from brain tissue of female *G. affinis*. Box plots indicate median, range and first and third quartiles. Dots indicate outliers. Significant differences ($p < 0.05$) are indicated with an asterisk.

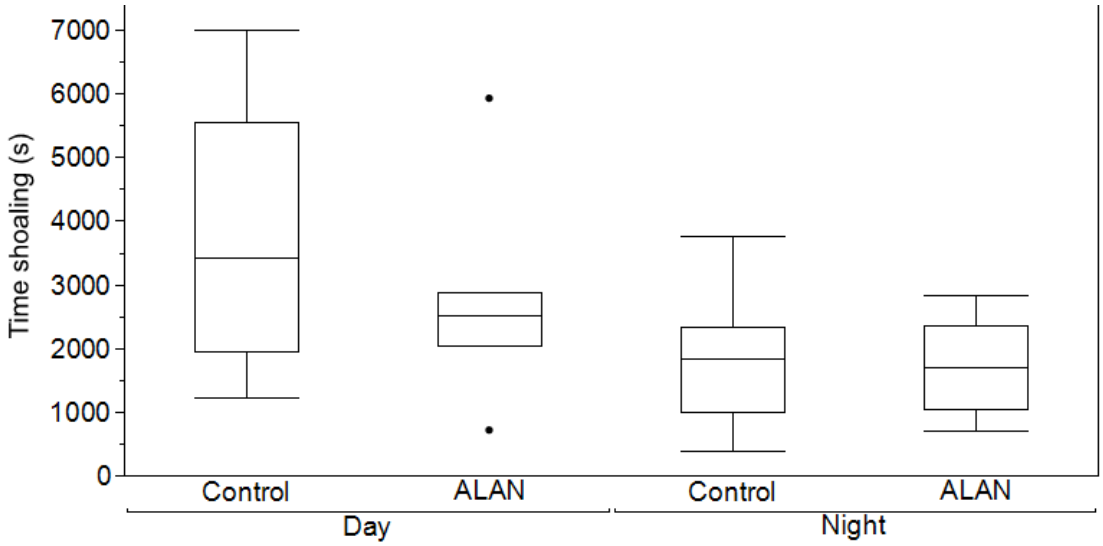

Fig. 6 The effects of ALAN on time spent shoaling of female *G. affinis* in the control (N = 15) and ALAN (N = 7) treatments in the day and night in the laboratory. Box plots indicate median, range and first and third quartiles. Dots indicate outliers.

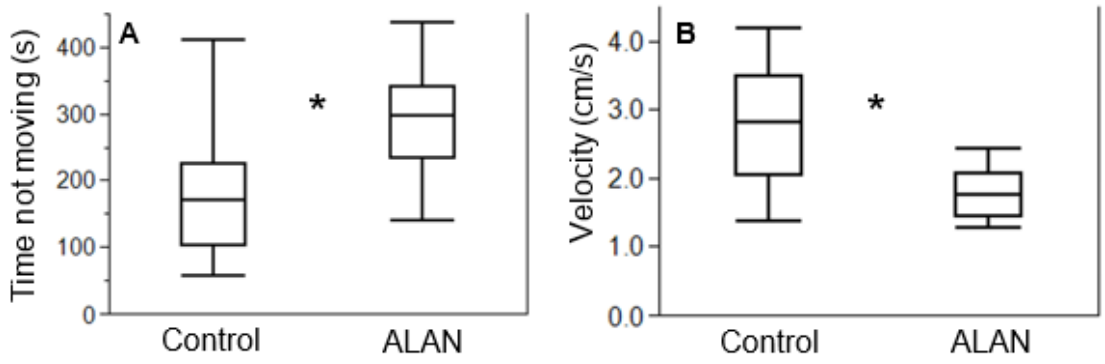

Fig. 7 The effects of ALAN on A) mean time spent not moving (± SE) and B) mean velocity (± SE) of individual female *G. affinis* (N = 29) in the laboratory. Box plots indicate median, range and first and third quartiles. Significant differences ($p < 0.05$) are indicated with an asterisk.

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