Exposure to artificial light at night in the wild leads to behavioral shifts in a freshwater fish (*Gambusia holbrooki*)

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ABSTRACT

Growth of urban areas represents a leading cause of biodiversity loss and can lead to altered phenotypes. Anthropogenic disturbances might not only cause negative behavioral consequences in animals, but also elicit adaptive behavioral responses via plasticity/learning or evolution. One pollutant universally linked with urbanization is artificial light at night (ALAN). ALAN causes widespread biological impacts, but we still know little about its ecological and evolutionary consequences, especially for aquatic organisms. Our field study examined ecological and phenotypic effects of ALAN in a diurnal, freshwater fish, the Eastern mosquitofish (Gambusia holbrooki). We did not observe consistent effects of ALAN on population demographics or body size. However, we observed a number of behavioral shifts—some matched a priori adaptive hypotheses. While most fish appeared to sleep during the night in populations unexposed to ALAN, we observed high incidence of nighttime activity in ALAN-exposed populations. Active fish swam at a much higher speed at night within ALAN-exposed populations, apparently extending feeding behaviors throughout the night. Based on activity patterns, one population—the one with the longest history and greatest magnitude of ALAN—even displayed a loss of diurnality. Females in ALAN-exposed populations showed reduced daytime feeding rates, perhaps resulting from successful nighttime feeding. Meanwhile, males exhibited higher rates of aggression and lower rates of sexual behaviors during the day in populations with greater magnitudes of ALAN. This could reflect proximate consequences of altered circadian rhythms, but could also involve an adaptive shift where males perform more difficult and risky mating behaviors under dim nighttime lighting where they might have higher success and lower risk from predators. For body condition and female daytime behaviors, we found evidence that mixing/gene-flow with un-exposed populations might have constrained adaptive divergence. Overall, we uncovered how one ubiquitous component of urbanization may have far-reaching consequences that extend beyond immediate, negative biological effects.

Keywords: Anthropocene, body condition, circadian rhythm, contemporary evolution, diel behaviour, light pollution, Poeciliidae, population demography, urbanization

Introduction

Due to the growth of human populations, cities are becoming more abundant and densely populated, with environments once deemed "natural" rapidly experiencing a variety of ecological changes as a result of urbanization. Current predictions estimate that two thirds of the human population will reside within urban areas by the year 2050 (United Nations, 2019). Organisms residing within these rapidly changing environments often experience negative consequences resulting in population declines and local extirpation of species (e.g. Aronson et al., 2014; Grimm et al., 2008; Li et al., 2022; McKinney, 2008; McKinney & Lockwood, 1999; Seto et al., 2012). Yet, these negative ecological consequences can be temporary or avoided altogether in some species owing to evolutionary rescue or adaptive phenotypic plasticity (e.g. Bell, 2017; Carlson et al., 2014; Caspi et al., 2022; Feiner et al., 2021; Gonzalez et al., 2013; Harmon & Pfennig, 2021; Snell-Rood et al., 2018). Not only do these circumstances offer a remarkable opportunity to improve our basic understanding of organismal responses to rapidly changing environments, but it has become imperative to understand the demographic and phenotypic changes experienced by these resilient organisms in the face of rapidly urbanizing environments to better mitigate negative human-caused impacts and reduce the rate of biodiversity loss (e.g. Carroll et al., 2014; Des Roches et al., 2020; Lambert & Donihue, 2020; Schell et al., 2021; Szulkin et al., 2020; Thompson et al., 2022).

While negative impacts of urbanization on biodiversity and population growth have been documented in diverse taxa, evidence for urban-induced changes in phenotypes is now rapidly accumulating (e.g. Alberti et al., 2017; Johnson & Munshi-South, 2017; Lambert et al., 2021; Szulkin et al., 2020), with important ecological, evolutionary, and conservation implications. Most research on this topic has so far focused on terrestrial organisms (e.g. Diamond & Martin, 2021; Perkin et al., 2011; Schell et al., 2021) even though urban impacts are known to be strong and pervasive in aquatic ecosystems (e.g. Paul & Meyer, 2001; Rosso et al., 2023; Vörösmarty et al., 2010; Wenger et al., 2009). Indeed, 60% of the world’s population lives within 100 km of coastal habitat and over half of the world’s population lives within three km of a body of freshwater (Bolton et al., 2017; Kummu et al., 2011). As expected, recent work has demonstrated that urbanization can cause rapid trait changes in aquatic species, including rapid, adaptive evolutionary shifts (e.g. Elizabeth et al., 2021; Brans & De Meester, 2018; Jenkins et al., 2021; Kern & Langerhans, 2018, 2019; Langerhans & Kern, 2020; Merckx et al., 2018). Yet this work has only just begun, and we currently know almost nothing about how one of the most novel and widespread aspects of urbanization—artificial light at night (ALAN)—might impact population ecology and phenotypes of aquatic organisms.

Anthropogenic light has been rapidly increasing in magnitude and spatial extent since the mid 20th century, with focused research on its global ecological impacts beginning in earnest only recently (Cinzano et al., 2001; Falchi et al., 2016; Sanders et al., 2020). Ecological studies have already uncovered widespread consequences of ALAN in disparate ecosystems and point to many areas in dire need of further research, including demographic and phenotypic consequences of ALAN in the wild (e.g. Altermatt and Ebert, 2016; Czaczkes et al., 2018; Gaston et al., 2015; Gaston & Bennie, 2014; Grose & Jones, 2020; Hölker et al., 2010; Hopkins et al., 2018; Longcore & Rich, 2004; Marangoni et al., 2022; Rich & Longcore, 2006; van de Schoot et al., 2024). Other than birds and sea turtles (e.g. Horton et al., 2023; McLaren et al., 2018; Witherington & Martin, 2003; Thums et al., 2016; Van Doren et al., 2017), we still know little in this regard. Because of the potential importance, prevalence, and ongoing expansion of ALAN along marine and freshwater ecosystems, researchers have recently begun investigating effects of ALAN in aquatic species other than sea turtles (e.g. Latchem et al., 2021; Marangoni et al., 2022; Moore et al. 2006; Nightingale et al., 2006; Pulgar et al., 2019). Moreover, unlike highly mobile or volant organisms that could potentially behaviorally avoid ALAN, some aquatic species exposed to ALAN have little ability to remove themselves from nighttime lighting owing to their life histories or their simple restriction to water. Yet, we are in the infancy of understanding the ecological and evolutionary consequences of ALAN in most aquatic systems and taxa, especially for freshwater fishes (Perkin et al., 2011).

Most fish show diel activity patterns, using periods of light and dark for foraging, predator avoidance, movement, and intraspecific interactions such as mating and social behaviors (Mehner, 2012; Pulgar et al., 2019). Sources of ALAN located near water sources can disrupt these behaviors as it interrupts the natural cycle of light under which these animals have evolved. In fact, ALAN represents one of the most novel of all anthropogenic stressors, as no lineage has experienced continuous lighting throughout the night across all seasons in its evolutionary history. This lack of experience with nighttime lighting suggests that the most common immediate consequence of ALAN may be negative behavioral impacts, but learned, adaptive responses are possible, as is adaptive evolution. So far, rather than examining the effects of prolonged exposure to ALAN in the wild, most research to date has experimentally manipulated light regimes in laboratory settings to largely establish a proof-of-concept that ALAN can have behavioral or physiological proximate effects on fish. For instance, Latchem et al. (2021), showed that bluegill sunfish (*Lepomis macrochirus*) experimentally exposed to ALAN exhibited lower daytime locomotor activity and higher nighttime swimming activity. In *Girella laevifrons*, an intertidal rockfish, experimental exposure to ALAN resulted in increased oxygen consumption and dramatically altered circadian and circatidal rhythms that caused higher overall activity levels (Pulgar et al., 2019). Trinidadian guppies (*Poecilia reticulata*) exposed to ALAN emerged more quickly from a refuge and spent more time in the open areas of their experimental tanks—behaviors that could expose them to increased predation risk in the wild (Kurvers et al., 2018). In smallmouth bass (*Micropterus dolomieu*), experimental exposure to ALAN induced increased activity during nest-guarding, which was posited to potentially have negative fitness consequences (Foster et al., 2016). Czarnecka et al. (2019) found that exposure to nighttime lighting increased nocturnal foraging rates on invertebrates by Eurasian perch (*Perca fluviatilis*). And Western mosquitofish (*Gambusia affinis*) experimentally exposed to ALAN had an overall lower swimming activity and lower glucose levels in the brain (Miner et al., 2021). Despite all this mounting evidence for effects of ALAN on activity level and foraging behavior in fish, we do not know how prolonged exposure to ALAN in the field may cause demographic, phenotypic, or evolutionary consequences. Clearly, we need field-based studies in urban aquatic systems to better understand the effects of ALAN on fish populations.

In this study, we tested how prolonged exposure to ALAN (~3-25 years) in nature has affected basic demography and key phenotypes in a common freshwater fish, Eastern mosquitofish (*Gambusia holbrooki*). These fish regularly inhabit urban streams, ponds, and lakes; some of these aquatic environments are exposed to varying degrees of ALAN. Eastern mosquitofish are visually oriented, relying primarily on their eyesight for foraging, social and sexual interactions, and escaping predation (Ward & Mehner, 2010). These small, livebearing fish have relatively short generation times (~2-3 per year) and are diurnal, relatively abundant, easily observed, and amenable to laboratory experiments. These features make them model organisms for testing the role of ALAN in causing altered ecology and evolution (e.g. population density, morphology, behavior).

Based on prior work, we hypothesized that *G. holbrooki* residing in urban freshwater bodies will experience adaptive phenotypic shifts in response to the altered selection regimes caused by prolonged exposure to ALAN. *Gambusia* fishes are known to exhibit rapid, ecologically driven phenotypic shifts via plasticity or evolution (e.g. Blanchard et al., 2024; Heinen-Kay et al., 2014; Jenkins et al., 2021; Langerhans, 2009; Moody & Lozano-Vilano, 2018; Riesch et al., 2015, 2018; Santi et al., 2020; Stearns, 1983; Stockwell & Weeks, 1999; Wood et al., 2022). We wished to test a series of predictions for adaptive differentiation in response to ALAN. To do this, we examined five pairs of populations, where each pair resided in a separate drainage and comprised an ALAN-exposed population and an ALAN-naïve population. We tested for ecological and phenotypic shifts by conducting measurements *in situ* in the field. If novel traits have enabled fish to tolerate or exploit ALAN, we should see 1) little-to-no differences in population demographics such as population density, age structure, or sex ratio, 2) little-to-no differences in body size or body condition, and 3) behavioral differences that could show parallel or non-parallel changes across drainages and reflect some combination of plasticity and evolutionary change. Alternatively, if negative impacts of ALAN are relatively long-lasting irrespective of possible behavioral shifts, we should observe in ALAN-exposed populations: 1) differences in demography, such as reduced population density or reduced juvenile recruitment, and/or 2) reduced adult body size or condition, especially in populations with shorter time periods of ALAN exposure. For adaptive, ALAN-induced behavioral shifts, we specifically predicted 1) elevated incidence of nighttime activity, 2) increased swimming speed and use of body and caudal fin periodic swimming during the night, 3) reduced daytime foraging and feeding rates owing to successful nighttime feeding, and 4) little change in daytime rates of aggression or sexual behaviors. These behavioral predictions largely derived from hypotheses of increased nighttime social and feeding activity in the presence of ALAN, as nighttime lighting should permit fish to effectively perform a range of visually guided behaviors that are important for fitness while experiencing reduced predation risk during the relatively dimly lit nights compared to the day (Nelson et al., 2022). We note two alternative possibilities for behavioral shifts: 1) if the increased energy expenditure of nighttime activity is not compensated for by nighttime energy acquisition in ALAN-exposed populations, then we could observe elevated daytime foraging and feeding rates, and 2) proximate effects of sleep deprivation and altered circadian rhythms / hormone dynamics could result in elevated aggression and/or reduced motivation for sexual behaviors during the day (e.g. Gutierrez-Perez et al., 2023; Van der Meer et al., 2004). And finally, if fish increase activity during the night in the presence of ALAN, they might also exhibit changes in spatial habitat use owing to altered risk/reward dynamics in dimly-lit nighttime conditions, but it is currently not clear how this might manifest.

METHODS

Study Sites

We investigated 10 urban populations of *G. holbrooki* in North Carolina, USA. All localities were within the Neuse River Basin near the city of Raleigh (in the city limits of Apex, Cary, Morrisville, and Raleigh) in an approximately 160 km^2 area. To test for differences between populations exposed and unexposed to ALAN, we carefully selected 5 pairs of sites, with each pair located in a separate drainage (Table 1; Fig 1). Each pair comprised a “Dark” site and an “ALAN” site in close proximity to one another. The Dark sites had never previously experienced substantial ALAN as far as we know, but sometimes had a small influence of distant lights from parking lots or buildings. The ALAN sites had all experienced artificial lighting from adjacent street lights, parking lots, or parking garages for at least the prior 3 years (Table 1). With one exception (Speight Branch), ALAN sites experienced the influence of ALAN across the entire site.

To focus on the effects of ALAN in this comparative study, we attempted to select sites that were similar in other aspects. For instance, all sites were permanent bodies of water with a pond-like habitat, having inlet and outlet streams. In each site, *G. holbrooki* was abundant and easily viewed from above the water’s surface. While we did not quantitatively survey aquatic communities, differences should be minimal across such similar habitats within these adjacent drainages (Olden et al., 2001; Zbinden et al., 2022), and we observed similar presence of fish species at all sites (e.g. bluegill, *Lepomis macrochirus*, largemouth bass, *Micropterus nigricans*).

Moreover, ALAN regime was not confounded with any measured water parameters (see Table A1).

Table 1

Summary of nighttime light exposure magnitude (lux) and history (years) for each study site, as well as the distance between paired sites within each drainage

Drainage	ALAN Status	Max Lux	Mean Lux	Years with ALAN	Pair Distance (m)
Speight Branch	ALAN	0.34	0.19	13	150
	DARK	0.00	0.00	0	
Walnut Creek	ALAN	5.33	2.38	16	200
	DARK	0.10	0.02	0	
Stirrup Iron Creek	ALAN	6.60	4.02	3	130
	DARK	0.00	0.00	0	
Brier Creek	ALAN	7.99	6.46	15	1,175
	DARK	0.08	0.04	0	
Williams Creek	ALAN	30.51	24.63	25	1,100
	DARK	0.05	0.02	0	

Note that values of 0.00 lux indicate light values less than 0.01 lux, and not an absolute absence of light.

To characterize the nighttime lighting at each site, we measured the light intensity and color of relevant artificial light sources during the night (23:00 – 01:30). We did this on the same day that we measured water-quality parameters and conducted demographic and activity surveys (see below). Subsequent light measurements for each site approximately one year later yielded nearly identical results (i.e. high repeatability of light-intensity measurements, intraclass correlation coefficient: $r = 0.98$, $P < 0.0001$). To estimate the light intensity from all relevant light sources, we walked the perimeter of each site to identify 5-7 locations that would accurately represent the typical (and maximum) light conditions experienced by *G. holbrooki* at night (typically in shallow, nearshore habitat). We then measured the illuminance near the water surface at each of these locations using an Extech EA30 (Nashua, New Hampshire) light meter, recording the lux to the nearest 0.01 units. While all Dark sites exhibited little to no influence of ALAN during the night, ALAN sites spanned a moderate gradient of light levels (Table 1). Thus,

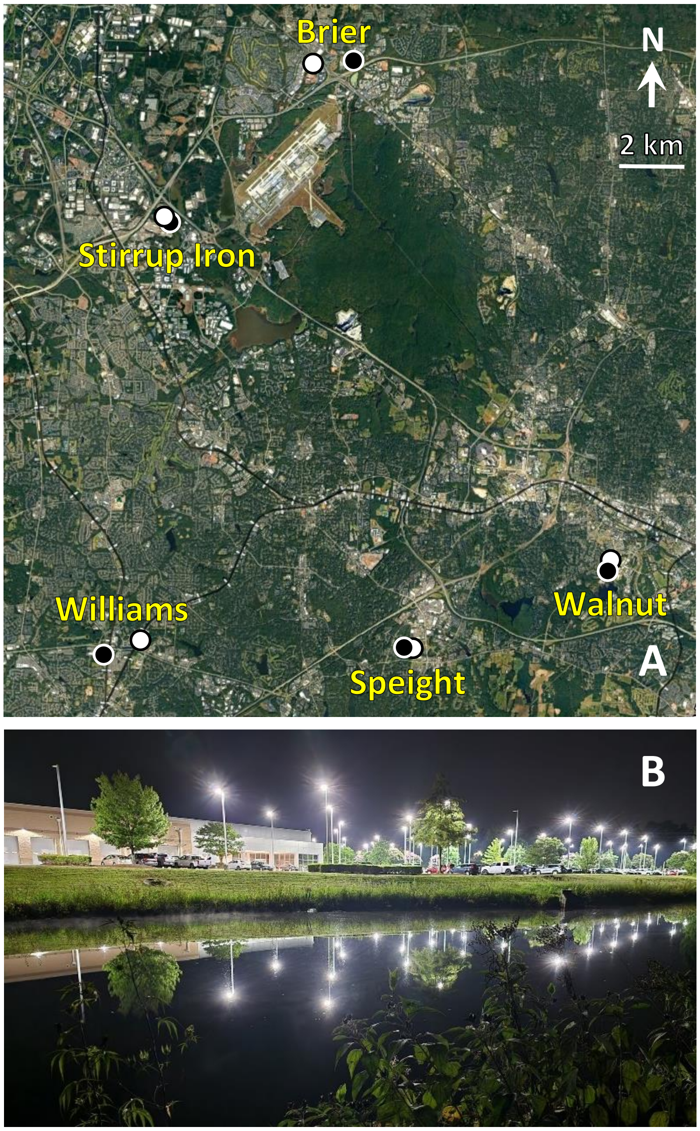

Figure 1. (a) Map of the 5 pairs of field sites near the city of Raleigh, North Carolina, USA, depicting the ALAN (white symbols) and Dark (black symbols) localities. Names indicate the five separate drainages. (b) Nighttime photograph of the ALAN site in the Williams Creek drainage.

fish in Dark sites experienced dark nights, with maximum brightness at the surface roughly equivalent to a clear, full moon (Kyba et al., 2017), but fish in different ALAN sites experienced either very dim nighttime lighting (Speight Branch) or moderate levels of nighttime lighting similar to lighting recommendations for most outdoor stairways, roadways, and pedestrian areas (recommendations range from 4-34 lux, Bureau of Street Lighting 2007). Moreover, ALAN sites varied in the timescale of exposure to ALAN, ranging from relatively short-term exposure (Stirrup Iron Creek) to more than two decades (Williams Creek) (Table 1). While light intensity could vary temporally at each site, variation is likely not particularly large for these sources of ALAN. To estimate the color (spectra) of all influential light sources at each ALAN site, we measured irradiance using a Jaz spectrometer (Ocean Optics, Dunedin, Florida). Near the water's edge, we pointed the end of an irradiance fiber optic cable toward all relevant light sources and saved spectra to a computer. Nearly all sources of ALAN at these sites derived from LED lighting with similar spectra properties, with only one site having additional types of lighting substantively influencing the site (Fig. A1). Thus, all ALAN sites experienced relatively similar spectra of lighting at night.

Population demographics, habitat use, and activity

To test for effects of ALAN on overall population demographics and diel patterns of habitat use and activity, we conducted randomized quadrat surveys of *G. holbrooki* during day and night at each site in the summer of 2020 (27 June – 6 August). *Gambusia* are most active during summer months because of longer photoperiods and warmer waters (Alcaraz & García-Berthou, 2007; Gao et al., 2019; Martin, 1975). At each site, we assigned 20 random 1m^2 quadrats spaced at least 3m apart and within 1m of the shoreline for observation. Previous work has found this method effective for *Gambusia* fishes in these habitat types (Araujo et al., 2014; Heinen-Kay et al., 2014; Riesch et al., 2015). Water depth was <0.5m for all quadrats. We selected this shallow, nearshore habitat type because of its prolific and obvious use by *G. holbrooki*, as well as its ease of visual assessment; however, these fish can utilize more offshore and slightly deeper environments as well. For each quadrat, a single observer (MRJ) counted the number and sex/age class (male, female, juvenile) of each *G. holbrooki*, and whether the quadrat contained active fish or not (0 vs. 1) during both the day and night survey at each site. We defined activity simply based on the presence of swimming behaviors, with inactive fish remaining stationary unless disturbed. Because *G. holbrooki* is a highly active, diurnal fish, we expected to only encounter inactive fish during the night. To visually observe fish, we carefully walked around the perimeter of each site, slowly approaching each quadrat to avoid any disturbance to the best of our abilities. During the night surveys, handheld flashlights with red filters were used for visual observations—we used red light to minimize disturbance of *G. holbrooki* (Fitzpatrick et al., 2013; Harvey et al., 2012; Widder et al., 2005). Lights were only turned on and pointed toward the water during quadrat observations. In each case, the daytime and nighttime surveys were conducted within 10-12 hours of each other during midday (~12:00-13:30) and approximately 2 hours past sunset (~23:00-0:30).

From these 400 quadrats (20 quadrats per 10 populations during both day and night), we calculated density (# individuals per m^2), age structure (# juveniles / # total), sex ratio (# females / # adults), and proportion active (# active / # total). Because some quadrats had either no adults or no fish present, less than 400 data points were examined for estimates of sex ratio (n = 366), as well as age structure and activity (n = 378). Density, age structure, and sex ratio were included as dependent variables in general linear models to test for effects of drainage, ALAN presence, day/night, and all interactions. In this way, we could test our hypotheses concerning how ALAN might influence these variables, whether effects of ALAN might be drainage-specific, whether ALAN might alter diel patterns (i.e. day/night differences), and whether ALAN might have altered diel patterns in drainage-specific manners. This means that in addition to testing population differences in demographic variables, we can detect diel shifts in habitat use; e.g. a more female-biased sex ratio observed in shallow, nearshore water during the day compared to the night implies that females may have used offshore or deeper waters more at night relative to males. To meet assumptions of normality of residuals, we $\log_{10}$-transformed density and arcsine square-root transformed age structure and sex ratio. We calculated η^2 as an estimate of effect size for each model term to evaluate relative importance of potential effects. To directly test for among-population associations between the magnitude of ALAN and demographic variables, we also tested the Pearson correlation between the maximum lux of each site and the day/night mean estimates of each demographic variable. Note that these results were qualitatively the same as those obtained through general linear mixed models testing for effects of maximum lux while including a random term for population throughout this study. Because we observed an intriguing pattern of sex-ratio differences between ALAN and Dark sites that could have resulted from density-dependent effects (see Results), we conducted separate analysis of covariance (ANCOVA) models for the daytime and nighttime data using population means that tested for variation in sex ratio attributable to density, ALAN presence, and their interaction. In this way, we could determine whether difference between ALAN and Dark sites could be statistically explained by a correlation with density, or alternatively persisted after statistically adjusting for variation in density.

To test variation in activity incidence, we did not conduct a general linear model as we did for demographic data because 15 of the 20 surveys exhibited no variation among quadrats (either all fish active or all fish inactive) and differences among surveys were apparent without statistical analysis. However, to specifically test the hypothesis that higher intensity of ALAN might lead to greater frequency of nighttime activity, we tested for a positive correlation between the proportion of quadrats with active fish during night surveys and the maximum lux of each site. To accomplish this, we used the non-parametric Spearman correlation because of the non-normality of the activity data. We conducted analyses in JMP software (v. 16.0, 2021, SAS Institute Inc., Cary, North Carolina, USA).

Locomotor Activity

To test whether ALAN influenced locomotor activity of *G. holbrooki*, we wanted to observe swimming behaviors and measure swimming speed and swimming style *in situ* in the absence of any possible human interference. To accomplish this, on the same days and nights when we conducted quadrat surveys, we recorded approximately eight videos during both day and night from overhead with an infrared-receptive camera (Panasonic HC-WXF991) at 3840 × 2160 resolution. To capture video of swimming fish and accurately measure their swimming behavior, we recorded videos in areas where *G. holbrooki* had been observed and restricted analysis to fish near the water surface (< 3 cm deep, limiting vertical displacement of fish). Video locations were separated by at least 5 m within sites. We mounted the video camera on a tripod, and in each case recorded video at 30 frames per sec for approximately five minutes in the absence of any nearby human from ~0.5 m above the water surface (~50 cm × 30 cm field of view). At the beginning of each video, a 3-cm laminated grid was placed just below the water surface for scale (*G. holbrooki* typically swim near the surface). During night recordings, we illuminated the field of view using the camera’s built-in infrared light, as well as two 850 nm infrared Souyos flashlights mounted onto the tripod. The videos provided clear views of unambiguously undisturbed fish for the measurement of swimming behavior during short time periods.

From the videos, we selected 30 fish for measurement that met the following criteria during each observation period at each site: active fish performing routine behaviors near the water surface, not directly interacting with conspecifics or other fish species, and in the camera view ≥ 5 sec. Within dark sites most fish were inactive during quadrat surveys, but video analysis revealed that some fish within these localities exhibited at least occasional active swimming behaviors. Because we could not clearly determine the sex or age class of all individuals in the videos, we instead selected fish of varying sizes from each site in an effort to provide a representative sample of fish from each population. For these fish, we measured 1) body size (standard length, SL) using tpsDig2 (Rohlf, 2017) with a screenshot from the video that provided a clear view of the unbent fish body, and 2) swimming speed during a 5-second video segment using DLTdv8 (Hedrick, 2008). SL was defined as the length between the tip of the snout and the posterior end of the vertebrae. In DLTdv8, we digitized the snout tip of the fish every 3rd video frame (i.e. every 0.1 sec) of the video segment (51 points spanning 50 time steps). This frequency of measurement resulted in smooth displacement by time graphs, indicating an adequate spacing with relatively low measurement error and little missing information between time steps. For each time step, we calculated swimming speed as distance traveled (mm) divided by time (sec). We then calculated the average swimming speed and coefficient of variation of swimming speed (CV = std. dev. / mean × 100) for each fish.

While average swimming speed was measured to capture the overall movement rate of fish, we calculated the CV of swimming speed to capture aspects of the swimming style. During the day, *G. holbrooki* typically exhibit body and caudal fin periodic propulsion during routine swimming (e.g. see Blake, 2004). However, during the night we noticed that active fish often displayed a burst-and-coast swimming style, typified by a rapid dart followed by a coast phase and a variable period of little motion. Moreover, while fish rarely come to a stop during routine swimming in the daytime, we observed stopping behaviors at night. This burst-and-coast behavior is characterized by high temporal variation in velocity compared to body and caudal fin periodic swimming, and thus the CV of swimming speed from video analysis should capture this variation (higher CV implies more burst-and-coast swimming and less body and caudal fin periodic propulsion).

Statistical analysis of average swimming speed and CV of swimming speed followed that described above for demographic variables, with the addition of body size (SL) as a covariate to statistically control for effects of body size. That is, we again conducted general linear models to test for effects of drainage, ALAN, day/night, and all interactions, and calculated η^2 as estimates of effect size. We $\log_{10}$-transformed swimming speed to meet assumptions for analysis. We observed two outliers—two individuals in Speight Branch ALAN site had extremely low average swimming speeds—and excluded these two fish from analysis. To test whether average daytime or nighttime locomotor activity was associated with maximum lux at night, we tested for Pearson correlations using population means.

Adult Daytime Behaviors

During the day, immediately following the demographic quadrat surveys, we conducted focal animal sampling (Bateson & Martin, 2021) along the shoreline to measure the frequency of common behaviors of *G. holbrooki*. All behavioral observations occurred during midday (~12:30

– 15:30) because focal sampling was not feasible at night. During each observation, a single observer (MRJ) visually followed one adult fish (see Table A2 for sample sizes) for a brief time (mean ± std. err. = females: 79.5 sec ± 1.5, males: 73.8 sec ± 1.8), recording the number of occurrences of four behaviors: foraging (prey inspections), feeding (bites), aggression (chasing or circling another fish), and sexual behaviors (male-female chase, mating attempt). For each fish, the observer attempted to follow the fish and record behavioral frequencies for approximately 90 sec, but view of the fish was frequently lost prior to this time point (e.g. lost in group of fish, briefly obscured by plant structure). To avoid observing the same individual multiple times, we took the following precautions: 1) we specifically selected fish of different sex or size for observations within a given ~3m^2 area, 2) we systematically moved in one direction along the shoreline between observations (avoiding returning to area already examined), 3) we covered a large area of each site, with most observations of the same sex conducted at least several meters from one another, and 4) performed all behavioral observations within a relatively short time frame at each site to prevent the potential for fish to move large distances (from 81 to 119 minutes per site). These methods and timeframes have been previously used successfully to characterize behaviors of poeciliid fish, including *Gambusia, in situ* (e.g. Heinen et al., 2013; Köhler et al., 2011; Kraft et al., 2016; Tobler et al., 2009). Here, we typically observed a number of behaviors during these observation periods, indicating the timeframe was biologically relevant (mean ± std. err. = 6.8 behaviors ± 0.3 per fish). Out of the 430 total fish we observed, only seven performed no behaviors during our observations.

For analysis, we first converted all behavioral observations to behavioral frequencies (# / min). We analyzed behaviors separately by sex because of the strong differences in all behaviors between the sexes. To account for non-independence of behavioral variables and reduce dimensionality, as well as generate trait axes that meet assumptions of general linear models, we conducted Principal Components Analysis (PCA) using the correlation matrix of the four behaviors separately by sex. We retained the first two PC axes in each case (those with eigenvalues > 1), accounting for 74.51% of variance in females and 72.08% of variance in males. For both sexes, PC 1 described variation in the rate of foraging and feeding behaviors, while PC 2 described a tradeoff where more positive scores were associated with higher aggression frequencies and lower frequencies of sexual behaviors and negative scores reflected lower aggression frequencies and higher frequencies of sexual behaviors (Table 2).

To test our hypotheses that ALAN might lead to changes in daytime *G. holbrooki* behavior, we constructed general linear models using the four relevant PC axes as dependent variables and drainage, ALAN status, and their interaction as independent variables. To assess whether the magnitude of nighttime lighting might influence daytime behaviors, we further examined among-population associations between average behavioral PC scores and maximum nighttime lux at each site using Pearson correlation. Because results for females suggested that the geographic proximity of paired sites might influence behavioral differences within drainages, we additionally conducted tests for associations between behavioral differentiation (difference in

Table 2
Loadings and variance explained for principal components analysis of behavioral frequencies for female and male *G. holbrooki* (n = 430) observed within the 10 focal populations

	Females		Males	
Behavior	PC 1	PC 2	PC 1	PC 2
Prey Inspection Freq.	**0.97**	-0.02	**0.93**	0.03
Biting Freq.	**0.97**	0.02	**0.88**	-0.09
Aggression Freq.	-0.21	**0.75**	-0.25	**0.72**
Sexual Behavior Freq.	-0.23	**-0.67**	-0.30	**-0.75**
Percent Variance Explained	49.36	25.14	44.91	27.17

Behaviors with strongest loadings on each PC axis in bold.

PC scores) and geographic distance for each sex using Pearson correlation. This specifically tested whether behavioral differences between ALAN and Dark sites tended to increase with increasing distance between the sites.

Body Size and Condition

To investigate whether ALAN might influence body size or condition of *G. holbrooki*, we examined approximately 20-30 adults of each sex from each site (Table A2), measuring their length, weight, and length-specific weight. We collected fish using seines and dip nets during 20 September – 2 October 2020, separately photographed and weighed each fish alive, and either returned them to their site of collection (6 populations) or housed them in the laboratory at North Carolina State University for subsequent experimentation in a separate study (4 populations). In all cases, we collected fish from each population within a given drainage within 24 hrs of each other. We attempted to collect and measure adults representative of the size distribution within each population. To measure SL, we placed each fish in a polypropylene beaker (9-cm diameter) with ~100 ml of water with a laminated ruler positioned on the bottom, and captured a photograph from above using a tripod-mounted DSLR camera (Canon T3i; Canon Inc., Tokyo, Japan) with a macro lens (Sigma 50 mm f/2.8 EX DG Macro; Sigma Corp., Ronkonkoma, New York). We then used tpsDig2 (Rohlf, 2017) to measure SL from the digital images. We measured the mass of each fish to the nearest 0.001 g by gently and briefly drying each fish in a small aquarium net and placing them into a 0.5L beaker filled with ~10ml of water that was tared on a balance (Adventurer model, OHAUS Corp., Parsippany, New Jersey, USA). We have previously demonstrated very high repeatability for these methods of measuring SL and mass in adult *G. holbrooki* (Langerhans et al., 2021).

For analysis of body size, we examined $\log_{10}$-transformed SL and $\log_{10}$-transformed mass. For analysis of body condition, we examined Fulton's K (weight relative to an expectation of simple isometric growth, $K = \text{mass}/\text{SL}^3 \times 100$). Note that Fulton's K was highly correlated with residuals from a linear regression of $\log_{10}$-transformed mass on $\log_{10}$-transformed SL ($r = 0.96$). To test whether ALAN influenced body size or condition of *G. holbrooki*, we constructed general linear models using these three variables as dependent variables, and drainage, ALAN, sex, and their interactions as independent variables. We calculated η^2 as estimates of effect size to evaluate the relative importance of model terms. We tested for among-population correlations of body size and condition with the magnitude of ALAN using Pearson correlation with sex-specific means. Because body condition might partially reflect overall health / fitness of adults, we tested for evidence that population mixing / gene flow might constrain adaptive differentiation by testing associations between body-condition differentiation among paired sites (mean condition in ALAN – mean condition in Dark) and geographic distance between sites using Pearson correlation separately by sex.

Ethical Note

This work was conducted with approval from the Institutional Animal Care and Use Committee of North Carolina State University (protocol 19-756-O) and the North Carolina Wildlife Resources Commission (license 20-SFC00250).

Results

Population demographics, habitat use, and activity

For population density, we found evidence for the influence of all model terms except the interaction between ALAN and day/night (Table 3). Based on effect-size estimates, the strongest influences on density involved drainage-specific effects of ALAN that partially depended on day/night. This means that ALAN tended to have heterogeneous effects on *G. holbrooki* density. Specifically, ALAN sites exhibited greater average density than Dark sites within three drainages (Speight Branch, Stirrup Iron, Brier Creek), while Dark sites never showed higher densities than ALAN sites within any drainage (Fig. 2a). Thus ALAN was sometimes, but not always, associated with higher population densities of *G. holbrooki*. Moreover, in ALAN sites we observed higher densities in shallow, nearshore water during the day than night within three drainages (Speight Branch, Walnut, Stirrup Iron; Fig. 2a). This diel change in density within shallow, nearshore habitat suggests that fish shifted offshore or generally spread out across these sites in a less aggregated spatial distribution at night. Within one drainage (Williams Creek), this pattern was observed within the Dark site, while the opposite pattern (higher density at night compared to the day) was exhibited in the ALAN site. Note that higher observed densities during the day cannot be explained as an artifact of lower detectability of *G. holbrooki* at night because this pattern was not observed in the majority of sites. We found no association between day or night density and maximum nighttime lux (both $P > 0.20$).

For age structure, we again found evidence for the influence of all model terms except the interaction between ALAN and day/night (Table 3). The strongest effects on the proportion of juveniles involved differences between drainages and drainage-specific effects of ALAN. We observed a greater average proportion of juveniles in ALAN sites compared to Dark sites within

Table 3. Results of general linear models examining variation in density, proportion of juveniles, and proportion of females attributable to effects of drainage, ALAN, day vs. night, and their interactions

Trait	Stat.	Drainage	ALAN	Day/Night	Drainage × ALAN	Drainage × Day/Night	ALAN × Day/Night	Drainage × ALAN × Day/Night	R^2
Density	*F*	14.82	77.09	22.26	22.81	4.93	1.03	18.46	0.48
	d.f.	4,380	1,380	1,380	4,380	4,380	1,380	4,380	
	P	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0007**	0.3108	**<0.0001**	
	η^2	**8.18**	**10.64**	3.07	**12.60**	2.72	0.14	**10.19**	
Prop. Juv.	*F*	20.73	39.15	4.30	14.34	4.57	0.45	3.22	0.37
	d.f.	4,358	1,358	1,358	4,358	4,358	1,358	4,358	
	P	**<0.0001**	**<0.0001**	**0.0388**	**<0.0001**	**0.0013**	0.5045	**0.0130**	
	η^2	**14.56**	**6.88**	0.76	**10.07**	3.21	0.08	2.26	
Prop. Fem.	*F*	1.89	33.41	1.74	8.70	0.83	8.86	2.49	0.22
	d.f.	4,346	1,346	1,346	4,346	4,346	1,346	4,346	
	P	0.1125	**<0.0001**	0.1879	**<0.0001**	0.5095	**0.0031**	**0.0428**	
	η^2	1.70	**7.52**	0.39	**7.83**	0.74	1.99	2.25	

P values ≤ 0.05 in bold, $\eta^2 \geq 5\%$ in bold.

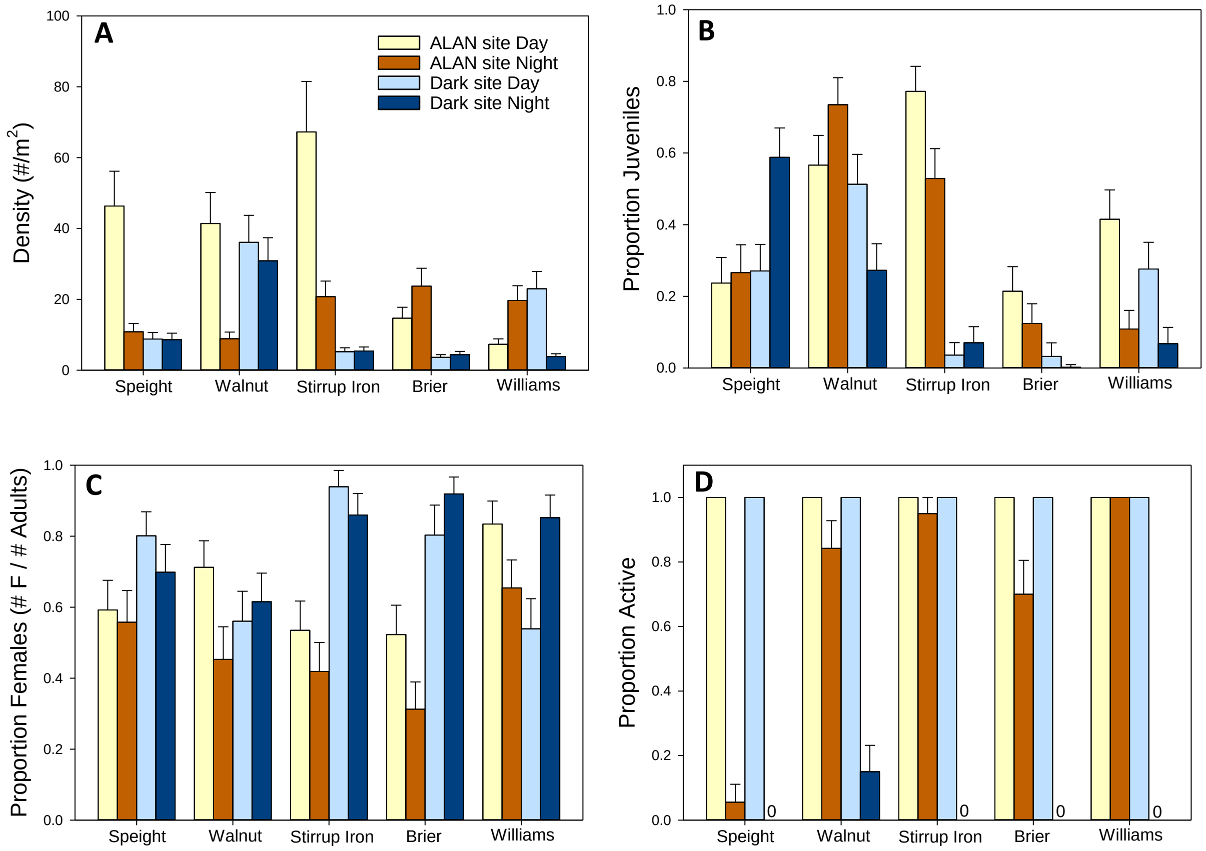

Figure 2. Variation among ALAN and Dark populations for both day and night across the five drainages in (a) density, (b) proportion of juveniles, (c) proportion of females, and (d) proportion of active *G. holbrooki* observed during visual surveys ("0" indicates no active fish observed). Mean ± 1 SE depicted.

two drainages (Stirrup Iron, Brier Creek); we never observed greater proportion of juveniles in Dark sites compared to ALAN sites within any drainage (Fig. 2b). This suggests that ALAN was sometimes, but not always, associated with a shift in age structure toward greater a proportion of juveniles. Further, while we did not observe strong differences in the proportion of juveniles between day and night in most sites, one drainage (Williams Creek) was characterized by a decrease in proportion of juveniles observed at night compared to the day (Fig. 2b). This suggests that within this drainage *G. holbrooki* may show an age-based diel shift in habitat use, with juveniles, but not adults (see results for density), tending to disperse at night toward a less aggregated spatial distribution. We observed no association between day or night proportion of juveniles and maximum nighttime lux (both $P > 0.40$).

We found that all model terms involving ALAN had an influence on sex ratio, but we found no evidence for effects of other terms (Table 3). The strongest effects involved drainage-specific ALAN influences on the proportion of females observed. Within three drainages (Speight Branch, Stirrup Iron, Brier Creek), we tended to observe more female-biased sex ratios in Dark sites compared to ALAN sites (Fig. 2c). Additionally, for most drainages we tended to observe more female-biased sex ratios during the day compared to the night in ALAN sites but not in Dark sites, although this was never especially strong (Fig. 2c). Together, these results suggest that ALAN was sometimes associated with an overall lower relative abundance of adult females in *G. holbrooki* populations, and more commonly associated with a moderate, sex-specific diel habitat shift. Using ANCOVAs to test whether these patterns might be explained by correlations with population density, we found that 1) mean daytime sex ratio was explained by a negative correlation with daytime density ($F_{1,6} = 7.97$, $P = 0.0302$) and not by ALAN ($F_{1,6} = 0.03$, $P = 0.86$) or their interaction ($F_{1,6} = 0.88$, $P = 0.38$), while 2) mean nighttime sex ratio exhibited a relatively lower proportion of females in ALAN populations ($F_{1,6}$ = 7.19, P = 0.0365), but showed no effects of nighttime density ($F_{1,6}$ = 2.54, P = 0.16) or the interaction term ($F_{1,6}$ = 0.30, P = 0.61). Note that results were similar if we substituted daytime density for nighttime density in the latter analysis. Thus, only nighttime sex ratios differed between ALAN and Dark sites after statistically adjusting for population density, indicating ALAN was associated with sex-specific diel shifts but not overall differences in sex ratio. Inspecting the density of each sex separately, there was a pattern for both sexes, where females tended to utilize nearshore habitat more during the day than night within ALAN sites (female density 31% higher in daytime), while males tended to utilize nearshore habitat more at night than the day (male density 14% lower in daytime). No association between day or night proportion of females and maximum nighttime lux was observed (both $P > 0.45$).

We found that all *G. holbrooki* observed during the day within all sites were active, but at night active fish were more frequently encountered in ALAN sites than Dark sites within all five drainages (Fig. 2d). This pattern was strongly apparent in all but one drainage: in the Speight Branch drainage, the ALAN site experienced an especially low lux from nearby ALAN (Table 1) and also did not show high frequencies of active fish at night. We never observed active fish during quadrat sampling at night within the Dark sites for 4 of the 5 drainages (Fig. 2d). Across all sites, average nighttime activity was positively associated with maximum lux (ρ = 0.92, P = 0.0001).

Locomotor Activity

We found that all model terms were important in influencing mean swimming speed, while most terms were influential for CV of swimming speed (Table 4). By far, the strongest effect was the diel effect, where *G. holbrooki* swam faster during the day than night (Fig. 3a) and exhibited a higher CV for swimming speed (i.e. greater burst-and-coast swimming style) at night compared to the day (Fig. 3b). Only in the ALAN site in the Williams Creek drainage—where nighttime lux was especially high—did we not observe a reduction in mean swimming speed and increase in CV during the night. We also found that active fish tended to swim faster in ALAN sites than Dark sites in most cases—during the day this pattern occurred in three drainages (Speight Branch, Stirrup Iron, Brier Creek), while during the night it was observed in four drainages (all but Speight Branch, which has a very low light intensity) (Fig. 3a). Meanwhile, CV of swimming speed showed highly heterogeneous associations with ALAN (Fig. 3b). Testing for among-site associations between locomotor activity and maximum lux at night, we found no association for daytime average swimming speed ($r = 0.47$, $P = 0.17$), a positive correlation for nighttime average swimming speed ($r = 0.64$, $P = 0.0447$), no correlation for daytime CV of swimming speed ($r = -0.24$, $P = 0.51$), and a suggestive negative correlation for nighttime CV of

Table 4
Results of general linear models examining variation in the mean and coefficient of variation in swimming speed attributable to effects of drainage, ALAN, day vs. night, their interactions, and the body-size covariate (SL)

Trait	Stat.	Drainage	ALAN	Day/Night	Drainage × ALAN	Drainage × Day/Night	ALAN × Day/Night	Drainage × ALAN × Day/Night	SL	R^2
Mean	*F*	25.96	43.14	360.60	21.44	12.67	6.73	25.91	69.37	0.55
Swim	*d.f.*	4,577	1,577	1,577	4,577	4,577	1,577	4,577	1,577	
Speed	*P*	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0097**	**<0.0001**	**<0.0001**	
	η^2	**8.05**	3.34	**27.95**	**6.65**	3.93	0.52	**8.03**	**5.38**	
CV (%)	*F*	12.70	3.47	295.36	32.56	19.09	1.09	24.13	5.32	0.53
Swim	*d.f.*	4,577	1,577	1,577	4,577	4,577	1,577	4,577	1,577	
Speed	*P*	**<0.0001**	0.0631	**<0.0001**	**<0.0001**	**<0.0001**	0.2978	**<0.0001**	**0.0215**	
	η^2	4.17	0.28	**24.24**	**10.69**	**6.27**	0.09	**7.92**	0.44	

P values ≤ 0.05 in bold, $\eta^2 \geq 5\%$ in bold.

swimming speed (*r* = -0.61, *P* = 0.0612). Thus, we observed evidence that higher intensity of nighttime lighting in the field was associated with faster nighttime swimming and a tendency for less burst-and-coast style swimming, at least under the highest levels of ALAN examined in this study.

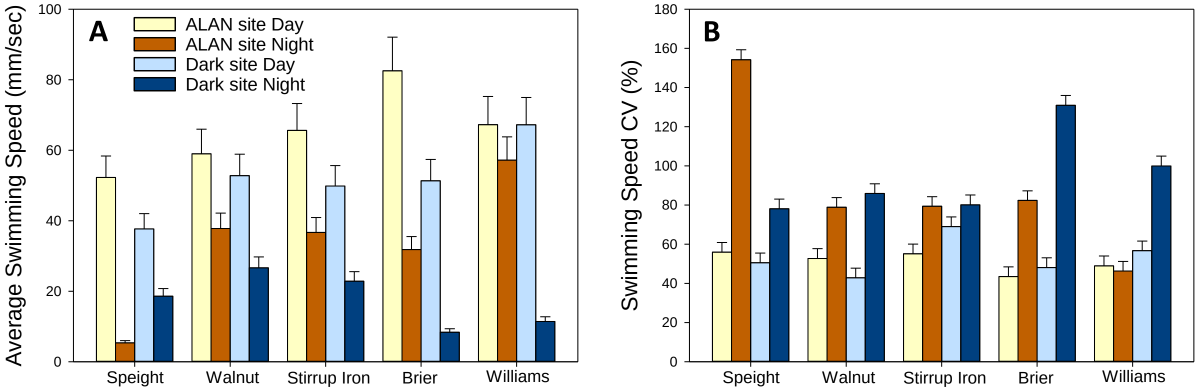

Figure 3. Variation in *G. holbrooki* (a) swimming speed and (b) coefficient of variation among populations during both day and night. Mean ± 1 SE depicted.

Adult Daytime Behaviors

For females, we found effects of ALAN on the first behavioral PC axis and drainage-specific ALAN effects on PC 2 (Table 5). Further examination of PC1 revealed that ALAN effects were only apparent within three drainages (Fig. 4a), consistent with the marginally non-significant interaction term. Overall, females exhibited behavioral differences between ALAN and Dark sites within every drainage but Stirrup Iron Creek. First, females exhibited similarly low daytime foraging and feeding rates within all ALAN sites, with Dark sites showing among-site variation (Fig. 4a). Second, females tended to show higher rates of aggression and lower sexual-behavior rates in ALAN sites within the Brier Creek drainage and more weakly within the Williams Creek drainage, but the opposite pattern was observed within the Walnut Creek drainage (Fig. 4b). We

Table 5

Results of general linear models examining variation in female and male *G. holbrooki* daytime behaviors attributable to effects of drainage, ALAN, and their interaction

Trait	Stat.	Drainage	ALAN	Drainage × ALAN	R^2
Female PC 1	*F*	1.96	20.53	2.20	0.15
	d.f.	4,205	1,205	5,205	
	P	0.1012	**<0.0001**	0.0704	
	η^2	3.25	**8.48**	3.63	
Female PC 2	*F*	3.18	0.76	2.88	0.11
	d.f.	4,205	1,205	5,205	
	P	**0.0146**	0.3840	**0.0237**	
	η^2	**5.52**	0.33	**5.00**	
Male PC 1	*F*	2.43	0.00	1.27	0.07
	d.f.	4,205	1,205	5,205	
	P	**0.0487**	0.9535	0.2829	
	η^2	4.42	0.00	2.31	
Male PC 2	*F*	1.09	6.25	0.68	0.06
	d.f.	4,205	1,205	5,205	
	P	0.3609	**0.0132**	0.6076	
	η^2	2.00	2.85	1.24	

P values ≤ 0.05 in bold, $\eta^2 \geq 5\%$ in bold.

found no clear association between maximum nighttime lux at each site and behavioral PC 1 scores ($r = -0.44$, $P = 0.20$) or behavioral PC 2 scores ($r = 0.24$, $P = 0.51$). When examining patterns for behavioral differentiation within drainages, we found that behavioral differences between paired ALAN-Dark sites tended to increase with increasing geographic distance between them ($r = 0.87$, $P = 0.0550$). Thus, smaller behavioral differences tended to occur among localities situated in close proximity to one another, such as within the Stirrup Iron Creek drainage.

Males showed fewer behavioral differences between ALAN and Dark sites than females, however they tended to display higher aggression rates and lower sexual behavior rates in ALAN sites within most drainages (Table 5, Fig. 4c,d). For the overall patterns most often observed for females (lower foraging/feeding, higher aggression, lower sexual-behavior rates in ALAN), males only strongly exhibited this pattern within the Williams Creek drainage. We found maximum lux at night was not associated with average behavioral PC 1 scores (*r* = -0.29, *P* = 0.41), but was positively associated with behavioral PC 2 scores (*r* = 0.79, *P* = 0.0069). Thus, males exhibited higher rates of aggression and lower rates of sexual behaviors during the day in populations that have experienced a greater magnitude of nighttime lighting. Behavioral differentiation between paired ALAN-Dark sites in males showed no clear association with the geographic distance between them (*r* = 0.66, *P* = 0.23).

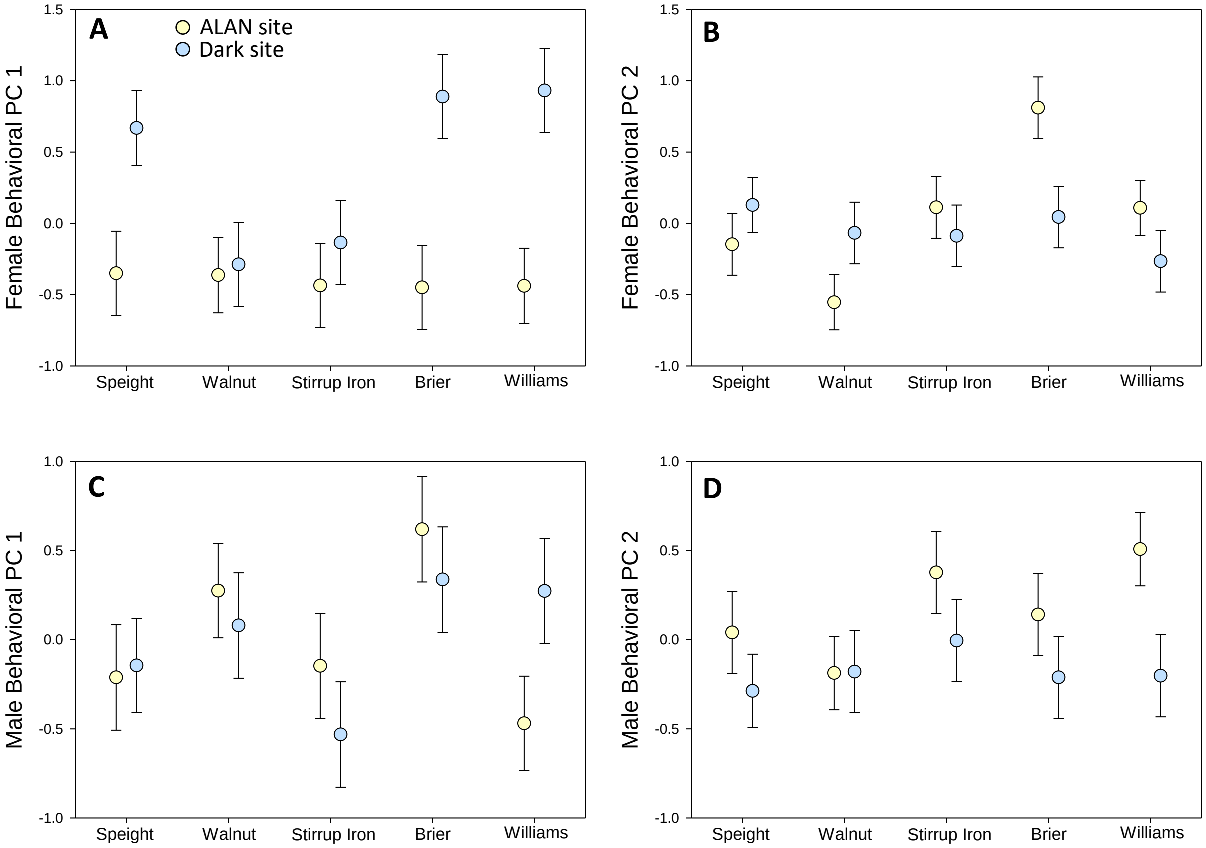

Figure 4. Variation among populations in daytime behavioral frequencies for (a,b) female and (c,d) male *G. holbrooki* along principal component axes. More positive scores of PC 1 describes increasing rates of foraging and feeding behaviors in both sexes. More positive scores of PC 2 describes increasing aggression frequency and decreasing rates of sexual behaviors. Mean ± 1 SE depicted.

Body Size and Condition

While we found non-trivial among-population variation and many influential factors, we found no consistent effects of ALAN on observed adult body size or condition of *G. holbrooki* in the field (Table 6; Fig. 5). Both estimates of body size yielded highly similar results, while patterns of body condition were distinctly different from those for body size. One clear pattern was that females were larger than males in all sites, as expected. For females, body size tended to be smaller in ALAN within the Brier Creek drainage (and weakly so for Speight Branch drainage), while it tended to be larger in ALAN in the Walnut Creek drainage. Female body condition tended to be lower in ALAN within the Speight Branch drainage (and weakly so for Stirrup Iron Creek drainage), but greater in ALAN within the Williams Creek drainage (and weakly so for Brier Creek drainage). For males, body size was larger in ALAN within the Walnut drainage, and trended toward that pattern within the Brier Creek and Williams Creek drainages, but the opposite trend was observed in the Stirrup Iron Creek drainage. Male body condition was higher

Table 6
Results of general linear models examining variation in body size and condition of *G. holbrooki* attributable to effects of drainage, ALAN, sex, and their interactions

Trait	Stat.	Drainage	ALAN	Drainage × ALAN	Sex	Drainage × Sex	ALAN × Sex	Drainage × ALAN × Sex	R^2
	d.f.	4, 420	1, 420	4, 420	1, 420	4, 420	1, 420	4, 420	
SL	*F*	8.79	1.01	13.01	1306.68	9.47	7.14	11.31	0.79
	P	**<0.0001**	0.3164	**<0.0001**	**<0.0001**	**<0.0001**	**0.0078**	**<0.0001**	
	η^2	1.81	0.05	2.68	**67.40**	1.95	0.37	2.33	
Mass	*F*	17.68	1.66	15.86	1311.58	9.20	6.15	11.91	0.78
	P	**<0.0001**	0.1987	**<0.0001**	**<0.0001**	**<0.0001**	**0.0135**	**<0.0001**	
	η^2	3.55	0.08	3.18	**65.77**	1.85	0.31	2.39	
Fulton's *K*	*F*	27.72	1.90	10.29	14.13	1.08	0.03	0.71	0.29
	P	**<0.0001**	0.1684	**<0.0001**	**0.0002**	0.366	0.8558	0.5873	
	η^2	**18.64**	0.32	**6.92**	2.37	0.73	0.01	0.48	

The *d.f.* were similar for all models. *P* values ≤ 0.05 in bold, $\eta^2 \geq 5\%$ in bold.

in ALAN within the Williams Creek drainage (and weakly so for Brier Creek drainage), but tended to be lower in ALAN within the Stirrup Iron Creek drainage. We found no associations between size or condition of either sex with maximum nighttime lux (all *P* > 0.16). However, we found evidence of a constraining effect of proximity to Dark sites on differentiation in body condition (correlation between geographic distance and difference in average condition; females: *r* = 0.82, *P* = 0.0924; males: *r* = 0.92, *P* = 0.0249). That is, *G. holbrooki* adults tended to have slightly lower condition in ALAN sites compared to Dark sites within drainages with short geographic distances between the sites, but higher condition in ALAN sites within drainages with longer distances between sites.

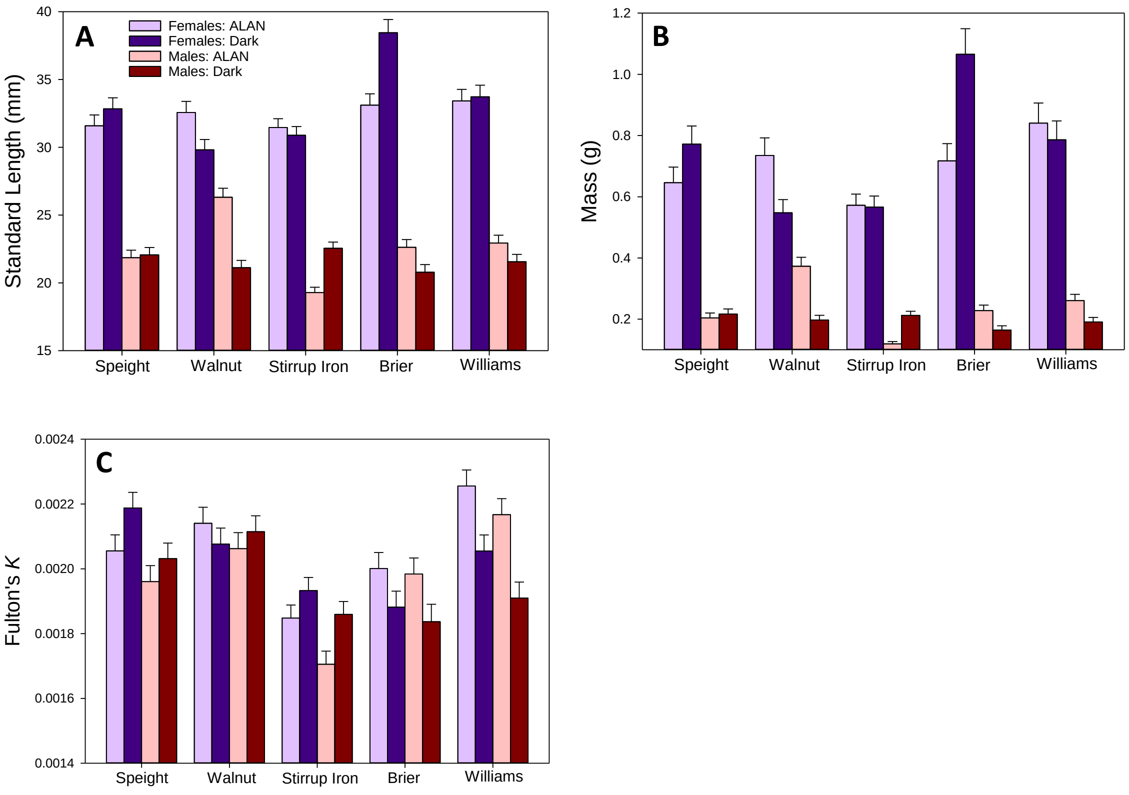

Figure 5. Variation among populations in (a) body length, (b) mass, and (c) condition in adult female and male *G. holbrooki*. Mean ± 1 SE depicted.

Discussion

Examining wild, urban populations of a freshwater fish, we uncovered evidence for a range of effects of human-caused nighttime lighting, with behavioral impacts generally stronger and more consistent than effects on population ecology, body size, or condition. Many effects of ALAN varied across drainages, and some of this variation was apparently attributable to variation in the magnitude of ALAN or the geographic proximity of populations within drainages. Regardless, we observed multiple effects of ALAN within every drainage examined, with many patterns matching our *a priori* predictions based on adaptive divergence. We suggest that ALAN can rapidly lead to phenotypic shifts in resilient organisms that persist in these urban environments, but that we still lack fundamental knowledge regarding the proximate and ultimate mechanisms underlying most changes, and population mixing / gene flow from nearby, unexposed localities can temper adaptive responses. These latter factors, and others (e.g. genetic [co]variances of traits, potentially rugged fitness surfaces, genetic drift), make it difficult to accurately predict the specific population-level outcomes of ALAN in all cases, even though the magnitude of differences between ALAN-exposed and ALAN-naïve populations within drainages were often similar to, or greater than, differences observed between drainages. Our findings indicate that long-term exposure to ALAN can even have such dramatic consequences that a “diurnal” organism may no longer exhibit diurnality.

Demographics and Habitat Use

Although effects of ALAN on population demography varied among drainages, we only observed patterns indicative of neutral to positive effects of ALAN on Eastern mosquitofish populations. This suggests that if ALAN has negative impacts on the population ecology of Eastern mosquitofish, it may be very short lived, and we did not observe it here. These patterns are consistent with *G. holbrooki* possessing traits or exhibiting rapid trait shifts that either adequately tolerated, or even benefited from environmental changes associated with ALAN. Considering the known negative proximate effects of ALAN in many taxa (e.g. Gaston et al., 2015; Gaston & Bennie, 2014; Horton et al., 2023; Latchem et al., 2021; Pulgar et al., 2019), a recent laboratory study using a subset of the populations examined here (Jenkins, 2023), and the behavioral differences observed in this study, it appears that *G. holbrooki* have partially contended with ALAN using adaptive behavioral changes.

The elevated population densities and greater proportion of juveniles observed in the presence of ALAN within some drainages may reflect greater population growth rates via increased fecundity or adult/juvenile survivorship. A direct effect of ALAN on reproductive output (e.g. testes size, brood size, reproductive timing) is possible (Baz et al., 2022; Dominoni et al., 2018; Durrant et al., 2018; Thawley & Kolbe, 2020), but indirect effects on reproductive output and survival may be more likely. This might occur for at least three reasons, as ALAN might 1) allow for increased food intake by extending foraging periods into the night (Cohen et al., 2011; Czarnecka et al., 2019; Dwyer et al., 2013; Fraser et al., 2004), 2) positively influence invertebrate prey abundance by directly altering invertebrate ecologies or by reducing densities of fish competitors (Bolton et al., 2017; Davies et al., 2012; Moore et al., 2006; Perkin et al., 2011), and 3) permit foraging to occur during time periods with lower associated predation risk (Cohen et al., 2011; Eckhartt and Ruxton, 2022; McNeil & Rodriguez, 1996; Nelson et al., 2022). Prior work in poeciliid fishes has shown that these three changes could lead to the demographic patterns observed here, as greater food availability / intake can increase fecundity and testes size, and lead to higher population densities with a greater proportion of juveniles, while lower mortality rates are also associated with higher densities and a higher proportion of juveniles (Heinen et al., 2013; Reznick et al., 1996; Riesch et al., 2020). This study provides evidence for elevated nighttime feeding in ALAN sites (see below), and we never observed nighttime predatory behavior of piscivorous fish during this study; but future work is needed to address mechanisms, as well as the reasons for among-drainage variation.

The more male-biased sex ratios observed during the day within several ALAN localities appeared to reflect a spurious effect of population density. During the day, we observed a lower proportion of females in cases with higher overall density—after statistically adjusting for this correlation, we detected no differences in daytime sex ratios between ALAN and Dark populations. While the cause of this density dependence is not known, some evidence for reduced proportion of females under higher density exists in another poeciliid fish (Zúñiga-Vega et al., 2012). Because prior work suggests that the female-biased sex ratios typical in poeciliid fishes (and commonly observed here) result from male-biased mortality (Snelson & Wetherington, 1980), patterns found in this study point toward increased survivorship of males in sites with higher population densities. Future research could examine how and why sex-specific mortality rates might co-vary with population density.

Meanwhile, nighttime sex ratios in shallow, nearshore habitat clearly differed between ALAN and Dark sites, regardless of population density, revealing unambiguous evidence for a sex-specific, ALAN-induced diel habitat shift. This sex-specific utilization of habitat types at night under dim lighting mostly resulted from an apparent movement of females offshore during the night, and less from males increasing nearshore habitat use at night. Offshore environments are not heavily utilized by *G. holbrooki* during the day, but perhaps nighttime lighting provides an opportunity for females to feed in areas that are typically dangerous during the day but experience reduced predation risk at night (see below). The open-water offshore environment also lacks complex plant structure which could provide shadows and refuge for prey, and this region could have elevated prey availability in the form of zooplankton or insects at the water surface. Moreover, light intensity might sometimes be slightly higher offshore where the sources of ALAN are not obscured by nearshore vegetation, providing more light for visually guided social and foraging behaviors, resulting in increased detection and consumption of prey. Likely owing to their elevated energetic demands, female mosquitofishes focus much more on foraging than males, which are more focused on mating (Heinen et al., 2013; Pärssinen et al., 2021). In this study, female *G. holbrooki* exhibited foraging and feeding rates during the day more than 3× that of males. This suggests, that diel habitat shifts of females toward offshore habitat at night might certainly involve a feeding motivation. Yet, females might also experience lower encounter rates with males offshore, reducing potential sexual harassment. While we did observe activity of *G. holbrooki* offshore at night within several ALAN localities (pers. obs.), we did not attempt to quantify offshore habitat use or identify the sex of fish swimming offshore.

To date, there is no prior work we are aware of that has examined the effects of ALAN on sex-specific habitat use. Previous work in guppies found they increased their use of open areas of tanks when exposed to ALAN in the laboratory (Kurvers et al., 2018); but that study did not examine behaviors during the night. Interestingly, sex-specific spatial aggregation diel shifts in fish have been noted in lemon sharks (*Negaprion acutidens*) where females shifted more offshore at night during winter months (Pillans et al., 2021). In freshwater catfish (*Tandanus tandanus*) females showed greater movement than males during the night (Koster et al., 2015). In roach (*Rutilus rutilus*), larger bodied females used more pelagic open habitat than smaller males

(Zak et al., 2020)—a pattern similar to what was observed here during the night. Future work should investigate the sex-specificity of ALAN-induced diel habitat shifts.

Diel Activity Incidence and Locomotor Activity

We initially hypothesized that ALAN would permit *G. holbrooki* to continue a range of activities into the night, especially feeding, resulting in elevated nighttime behavioral activity. Matching these *a priori* predictions, we found that populations with ALAN tended to show both a greater incidence of nighttime activity and higher nighttime swimming speeds. Furthermore, populations with a greater magnitude of nighttime lighting tended to show greater nighttime activity, suggesting that lighting per se influenced the level of activity in these visually-oriented fish. These differences were some of the strongest and most consistent effects of ALAN observed in this study. Our findings are consistent with the hypothesis that *G. holbrooki* takes advantage of nighttime lighting to extend feeding into the night because they can more effectively detect and consume prey under these artificially elevated levels of light. If ALAN was associated with increased prey abundance or quality, this could have reinforced this observation. This is also consistent with our anecdotal observations that most behaviors occurring during the night were foraging/feeding behaviors, as well as with the known increased nighttime feeding rates on invertebrates found in Eurasian perch in response to ALAN exposure (Czarnecka et al., 2019). Nighttime foraging as a result of ALAN has also been observed in other diurnal taxa such as arthropods (McMunn et al., 2019) and birds (Leveau 2020; Silva et al., 2017). And Batty et al. (1990) found that swimming speed in herring (*Clupea harengus*) increased with increasing light intensity, but only when they were feeding in manners similar to those used by *G. holbrooki* (i.e. biting, not filter-feeding). Moreover, if elevated nighttime activity largely reflected nighttime foraging activity, then we might expect to observe greater nighttime activity under conditions of higher resource competition. In line with this expectation, examining among-population associations with density in this study we found that both incidence of nighttime activity and nighttime swimming speed showed evidence of a positive association with nighttime density ($\rho = 0.72$, $P = 0.0191$; $r = 0.57$, $P = 0.0835$, respectively). Thus, we suggest that increased nighttime activity in populations with ALAN partially reflected an adaptive foraging shift in *G. holbrooki*.

Elevated nighttime activity may, however, come at an energetic cost—e.g. resulting in altered daytime behaviors or reduced body condition (see below)—if it isn't compensated for by increased energetic intake, such as profitable nighttime feeding (Fraser et al., 2004). For instance, rockfish experimentally exposed to ALAN exhibited increased oxygen consumption associated with their elevated activity levels (Pulgar et al., 2019). We observed fish consuming prey during the night via direct visual observations and in nighttime video recordings, indicating that *G. holbrooki* can successfully forage at night. Fraser et al. (2004) found that nighttime feeding in Trinidadian guppies, another "diurnal" poeciliid fish, was as profitable as daytime feeding even in the absence of ALAN. While *G. holbrooki* seem to seldom forage at night in Dark sites examined in this study, when they do forage at night in ALAN sites, they could be effective predators and compensate for their higher nighttime energetic expenditure. If so, fish in ALAN localities could require less daytime feeding to acquire similar or greater overall energetic inputs from food resources than fish in Dark sites which feed much less during the night (see below). Indeed, recent work in a subset of the populations studied here found that *G. holbrooki* from ALAN sites have even higher foraging performance during the night under ALAN than they do during the day, and have much higher nighttime foraging performance than fish from Dark sites (Jenkins, 2023).

While nighttime activity in *G. holbrooki* could increase rates of food consumption, it could also expose the fish to nighttime predation risk. We did not quantify activity of fish predators or predation rates in this study, but our observations suggest greatly reduced predation risk during the night owing to apparently strongly reduced activity of piscivorous fish and birds. That said, future work should investigate predation risk because ALAN (especially high intensity ALAN) can sometimes increase nighttime predation risk (Becker et al., 2013; Nelson et al., 2021; Sanders et al., 2021), perhaps centered along light-dark boundaries. Importantly, the ALAN sites studied here had little-to-no dark regions during the night—the only exception being the Speight Branch drainage, which also exhibited the lowest intensity of ALAN, lowest incidence of nighttime activity, and lowest nighttime swimming speeds among ALAN sites.

In most populations, we observed a strong diel shift in swimming speed and style, with slower average speeds and a more burst-and-coast style observed during the night than during the day. This likely reflects the greater overall activity and more diverse behaviors performed during the day than night in *G. holbrooki*. However, this diel shift was not observed in one population, the ALAN locality in the Williams Creek drainage, which had the highest light intensity and longest time of exposure to ALAN of any sites examined in this study. Fish in only this population showed similar activity incidence, swimming speed, and swimming style during the day and night. This population was also the only one that showed evidence in *both* sexes for reduced daytime foraging/feeding rates, elevated aggression frequency, and reduced rates of sexual behaviors compared to its paired Dark site within the drainage. These results suggest that under long-term exposure to relatively bright ALAN, diurnality may be lost in some “diurnal” organisms, even without a shift to nocturnality. While human disturbances are known to alter diel activity patterns in many taxa (e.g. Bonnot et al., 2020; Gaynor et al., 2018; Lee et al., 2024; Ordiz et al., 2014), this is the first case to our knowledge of a specific human-caused factor (ALAN) leading to the apparent loss of diurnality in the wild. Such a dramatic change in diel behavioral patterns could reflect a combination of phenotypic plasticity and evolutionary change, and have important ecological and evolutionary consequences.

The fact that populations with ALAN often showed faster swimming speeds than Dark sites during the *day* (within 3 drainages) could reflect altered circadian rhythms. Higher activity during the day has also been observed in rockfish and smallmouth bass experimentally exposed to ALAN (Foster et al., 2016; Pulgar et al., 2019). In contrast, experimental exposure to ALAN appears to lower daytime locomotor activity in bluegill sunfish (Latchem et al., 2021), and even in prior studies in mosquitofishes (Barzegar et al. 2022; Miner et al., 2021). However, the latter studies examined a different variable, time spent resting, and Miner et al. (2021) found conflicting results between an aquarium and mesocosm experiment in *G. affinis*. Contradictory evidence regarding effects of ALAN on daytime activity also comes from diverse taxa, with increased, decreased, and unaffected daytime activities documented (e.g. Baz et al., 2022; Duarte et al., 2019; Dwyer et al., 2013; Finch et al., 2020; Lynn et al., 2021; Taylor et al., 2022; Touzot et al., 2019; Ulgezen et al., 2019), but these studies have almost exclusively examined experimental exposure to ALAN over short time periods, not wild populations exposed to ALAN for generations. Based on our findings regarding feeding behaviors and body condition, elevated daytime swimming speeds in ALAN sites were not related to increased feeding rates or altered body condition. Moreover, daytime locomotor speed was not density-dependent, as we observed no correlation between average daytime swimming speed and average daytime density (r = 0.27,

$P = 0.46$). The underlying proximate and ultimate causes of ALAN-associated changes in daytime locomotor activity requires further investigation.

Daytime Behavioral Frequencies

ALAN was sometimes associated with decreased daytime foraging and feeding rates in female *G. holbrooki*, while males only exhibited this pattern in one locality. Notably, foraging and feeding rates were uniformly low for females in all ALAN populations. Perhaps elevated nighttime feeding in these sites allowed these fish to maintain low foraging activities during the day. Indeed, there was a suggestive trend for populations with greater average nighttime swimming speed to exhibit lower average daytime foraging/feeding rates in females ($r = -0.55$, $P = 0.0982$). As mentioned earlier, female mosquitofishes exhibit more resource-associated behaviors than males, and this is consistent with the notion that females may respond more strongly to ALAN in their feeding behaviors. In this study, we can rule out that reduced daytime feeding rates simply resulted from altered population densities, as we did not find any evidence of positive associations between average female or male PC1 scores and density (females: $r = -0.46$, $P = 0.19$; males: $r = 0.14$, $P = 0.70$). To date, we have little knowledge about the effect of ALAN on daytime foraging and feeding behaviors. There is some evidence of reduced daytime feeding in response to experimental exposure to ALAN in a marine mollusk (Manriquez et al., 2021). Meanwhile, Dwyer et al. (2013) found that daytime foraging was not associated with ALAN in a migratory shorebird (*Tringa tetanus*). More research is needed on this topic.

Males in ALAN sites with greater magnitudes of nighttime lighting tended to show higher rates of aggression and lower rates of sexual behaviors during the day, while females showed heterogeneous patterns for these behaviors. This behavioral shift in males could reflect daytime consequences of nighttime activity and altered circadian rhythms. In line with this hypothesis, populations with higher average PC 2 scores in males (higher aggression, lower sexual behavior) had greater nighttime activity levels ($\rho = 0.77$, $P = 0.0093$). Exposure to ALAN can affect aggression in many taxa (e.g. Carvalho et al., 2013; Li et al., 2024; Valdimarsson & Metcalfe, 2001; Van der Meer et al., 2004). ALAN exposure also alters sexual behaviors in many taxa (e.g. Gutierrez-Perez et al., 2023; McLay et al., 2018; Simonneaux & Bahougne, 2015; Touzot et al., 2020; Van Geffen et al., 2015). Effects vary, but increased aggression and decreased sexual motivation or reproductive success has been commonly reported, presumably involving several possible proximate mechanisms associated with altered circadian rhythms and sleep deprivation. ALAN can also disrupt organismal processing of photoperiod cues, leading to altered season-dependent sexual behaviors (Davies et al., 2023; Russart & Nelson 2018). Lower rates of sexual behaviors during the day under ALAN conditions could also reflect a plastic or evolved shift in diel patterns of risky mating behaviors—e.g. under increased levels of nighttime lighting, males could attempt more matings during the night instead of the day, as resistant females may more easily be overcome by males under dim lighting and the behaviors may have lower probability of attracting the attention of predators. We did observe mating behaviors during the night in ALAN-exposed populations, but quantitative study of these rates and their success requires future investigation. More research is needed to understand the mechanisms underlying these sex-specific behavioral changes associated with prolonged exposure to ALAN.

The degree to which behavioral differences among populations reflect plasticity or genetic differentiation is unknown, but both sources of variation are likely involved. If trait changes mostly reflected evolutionary change, we might expect an association between population trait values and time of exposure to ALAN. But evolutionary change need not show such an incremental change over this time period, and in this study, sites with longer exposure to ALAN also tended to have higher intensity of ALAN, making it difficult to tease apart those effects. Our findings for female foraging and feeding rates support a role for evolutionary change in explaining some of these patterns, as the magnitude of behavioral differences between ALAN and Dark sites within drainages increased with increasing geographic distance between sites. This suggests that gene flow between populations might constrain adaptive divergence, or that movement of individuals between locations might prevent adaptive plasticity (Garant et al., 2006; Langerhans et al., 2003). We find the likelihood of the former explanation to be greater than the latter in this case, as regular movement of individuals between these locations seems less likely than occasional movement that can influence gene flow. Additionally, a recent common-garden experiment using a subset of the populations investigated here has demonstrated evolutionary divergence between ALAN and Dark sites in multiple drainages for multiple traits (Jenkins, 2023), and thus at least some of the behavioral differences observed in the wild here may reflect evolution and not only plasticity. Future work is needed to directly address this topic.

Body Size and Condition

ALAN did not simply induce negative effects that resulted in lower body size or condition in *G. holbrooki*, as we observed inconsistent variation among ALAN and Dark sites. Differences in size and condition between paired sites were often observed for both sexes, but the directionality of differences went in both directions (e.g. sometimes smaller in ALAN, sometimes larger in ALAN). Effects of ALAN on body size may generally be species-specific, population-specific, dose-dependent, or time-dependent, as so many factors can affect body size. To date, experimental work has found that ALAN can sometimes lead to increased growth / body size (e.g. Batra et al., 2019; Borniger et al., 2014; Durrant et al., 2018; Fonken et al., 2010; Malek & Haim, 2019; Thawley & Kolbe, 2020) and sometimes lead to reduced growth / body size (e.g. Arvedlund et al., 2000; Boldogh et al., 2007; Dananay & Benard, 2018; Raap et al., 2016; Schligler et al., 2021; Villamizar et al., 2011).

The observed effect of geographic distance on the nature of body-condition differentiation between paired ALAN and Dark sites suggests a constraining effect of gene flow on adaptive divergence in ALAN populations. *Gambusia holbrooki* adults tended to have higher condition in ALAN sites compared to Dark sites within drainages with a farther distance between the sites, but lower condition in ALAN sites within drainages with a short geographic distance between sites. Populations exposed to ALAN might experience strong selection for altered phenotypes, but gene flow from nearby populations unexposed to ALAN can inhibit much adaptive evolution. This scenario can result in reduced body condition within ALAN sites. Meanwhile, ALAN populations with less gene flow from populations unexposed to ALAN can more readily adapt to ALAN conditions, potentially leading to increased body condition as these fish more effectively take advantage of the ability to feed and mate during the night. Future work can address these hypotheses.

Conclusions

Overall, we found that *G. holbrooki* regularly exposed to ALAN for years in the wild exhibited multiple phenotypic shifts within each of multiple drainages. Rather than reflecting negative consequences of human-altered conditions, most of these changes matched our *a priori* predictions of adaptive divergence, and some shifts must have occurred quite rapidly (all within 3-25 years; ~6-75 generations). This adds to the growing evidence for the role of human activities in driving adaptive trait changes in resilient organisms persisting in the human-altered environments. The largest differences in behaviors between ALAN and Dark sites within drainages typically occurred in cases with brighter ALAN, suggesting stronger selection under higher magnitudes of ALAN. The only clear evidence for negative consequences of ALAN came from the lower body condition observed in two drainages, but these two ALAN sites had the closest proximity to Dark sites—gene flow with un-exposed populations may have constrained adaptive responses to ALAN. Our study was the first of its kind to document demographic and behavioral effects of ALAN on an aquatic organism, and highlights the importance of conducting field studies to better understand the natural consequences of this pervasive pollutant.

Author Contributions

To be added after double-blind review.

Data Availability

Data for this study are either provided in the text, supplementary material, or (will be) deposited in the Dryad Digital Repository.

Declaration of Interest

We declare we have no competing interests.

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Supplementary Material

Supplementary material associated with this article is available, in the online version, at XXX.

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Appendix

Table A1
Average measurements of water-quality parameters taken from undisturbed water at ~20cm depth during the day and night at each study site

Drainage	ALAN Status	Date	Times	Water Temp (˚C)	pH	Turbitiy (NTU)	Salinity (ppt)	Conductivity (uS)
Speight Branch	ALAN	16-Jul-20	11:55, 22:45	27.6	8.24	14.11	0.1	130.4
	DARK	3-Jul-20	12:30, 22:45	30.9	9.76	18.99	0.0	70.1
Walnut Creek	ALAN	27-Jun-20	12:40, 23:10	27.8	8.62	3.10	0.1	127.4
	DARK	19-Jul-20	11:50, 22:50	26.5	7.76	4.41	0.1	156.3
Stirrup Iron Creek	ALAN	14-Jul-20	12:00, 0:20	32.2	9.11	2.12	0.1	246.8
	DARK	13-Jul-20	12:00, 23:00	27.6	8.63	5.91	0.1	128.6
Brier Creek	ALAN	2-Aug-20	12:00, 23:00	31.1	8.97	0.36	0.1	102.4
	DARK	6-Aug-20	12:05, 23:10	29.3	9.04	8.47	0.0	59.6
Williams Creek	ALAN	27-Jul-20	11:55, 22:55	32.6	9.13	15.05	0.0	59.2
	DARK	28-Jul-20	12:25, 23:00	31.7	8.49	3.92	0.0	93.9

Water temperature, salinity, and conductivity were measured using a YSI Pro2030. We measured pH using a Dr.meter PH100, and turbidity using a Sper Scientific 860040.

Table A2
Sample size information for the various components of the study during the day (D) and night (N), when applicable, for each population of *G. holbrooki*.

Drainage	ALAN Status	Quadrat Surveys	Locomotor Activity	Daytime Behaviors		Body Size/Condition	
				Females	Males	Females	Males
Speight Branch	ALAN	20 D / 20 N	30 D / 30 N	20	20	20	20
	DARK	20 D / 20 N	30 D / 30 N	25	25	21	21
Walnut Creek	ALAN	20 D / 20 N	30 D / 30 N	25	25	20	20
	DARK	20 D / 20 N	30 D / 30 N	20	20	20	20
Stirrup Iron Creek	ALAN	20 D / 20 N	30 D / 30 N	20	20	30	30
	DARK	20 D / 20 N	30 D / 30 N	20	20	30	31
Brier Creek	ALAN	20 D / 20 N	30 D / 30 N	20	20	20	20
	DARK	20 D / 20 N	30 D / 30 N	20	20	20	17
Williams Creek	ALAN	20 D / 20 N	30 D / 30 N	25	25	20	20
	DARK	20 D / 20 N	30 D / 30 N	20	20	20	20

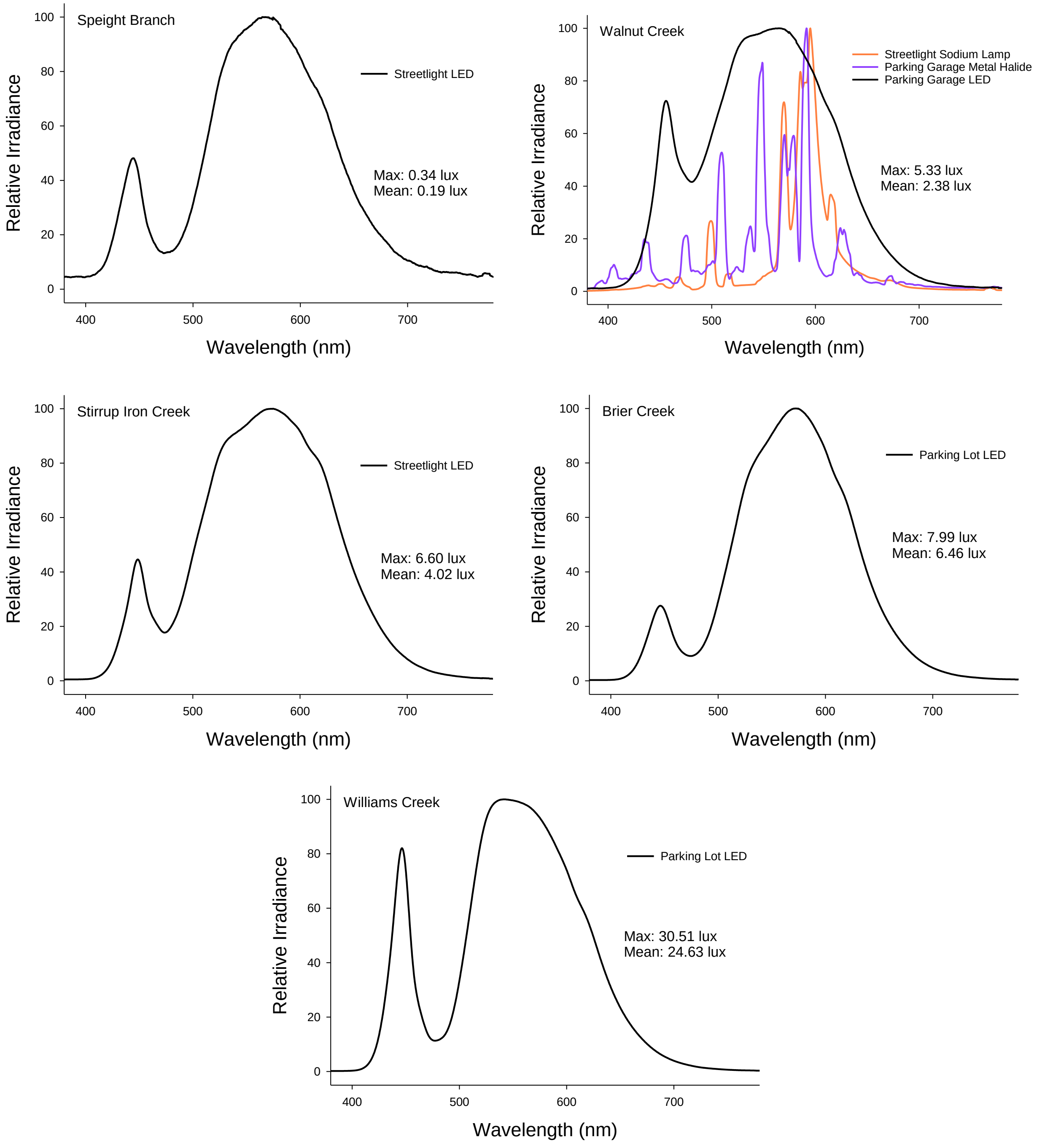

Figure A1. Spectra of ALAN light sources as measured from near the shoreline at each of the five ALAN localities examined in the study. Multiple measurements of multiple light sources were taken in all cases (mean standardized spectra depicted), but spectra were very similar among lights at each site with the exception of the ALAN site within the Walnut Creek drainage. Thus, we only present multiple spectra for the latter drainage, where distinct types of light sources were present. In all cases, including Walnut Creek, LED lighting provided the dominant type of light source at night affecting the nighttime light conditions of these *G. holbrook*i populations.

Highlights

- Artificial light at night (ALAN) has led to adaptive behavioural changes in a fish
- Fish are much more active at night under ALAN, and altered their daytime behaviour
- Females exhibited a habitat shift at night under ALAN
- Changes in condition and female daytime behaviour may be constrained by gene flow
- The population under the greatest intensity of ALAN displayed a loss of diurnality

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Supplementary Interactive Plot Data (CSV)

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