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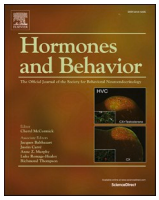

Noise and light pollution elicit endocrine responses in urban but not forest frogs

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ABSTRACT

Urban areas are characterised by the presence of sensory pollutants, such as anthropogenic noise and artificial light at night (ALAN). Animals can quickly adapt to novel environmental conditions by adjusting their behaviour, which is proximately regulated by endocrine systems. While endocrine responses to sensory pollution have been widely reported, this has not often been linked to changes in behaviour, hampering the understanding of adaptiveness of endocrine responses. Our aim was, therefore, to investigate the effects of urbanisation, specifically urban noise and light pollution, on hormone levels in male urban and forest túngara frogs (*Engystomops pustulosus*), a species with reported population divergence in behaviour in response to urbanisation. We quantified testosterone and corticosterone release rates in the field and in the lab before and after exposure to urban noise and/or light. We show that urban and forest frogs differ in their endocrine phenotypes under field as well as lab conditions. Moreover, in urban frogs exposure to urban noise and light led, respectively, to an increase in testosterone and decrease in corticosterone, whereas in forest frogs sensory pollutants did not elicit any endocrine response. Our results show that urbanisation, specifically noise and light pollution, can modulate hormone levels in urban and forest populations differentially. The observed endocrine responses are consistent with the observed behavioural changes in urban frogs, providing a proximate explanation for the presumably adaptive behavioural changes in response to urbanisation.

1. Introduction

Anthropogenic activities, including urbanisation, are rapidly changing abiotic and biotic conditions (Grimm et al., 2008; James, 2018). These novel environmental conditions alter natural and sexual selection pressures, and can cause populations to decline or to adapt (Johnson and Munshi-South, 2017; McKinney, 2006; McKinney and Lockwood, 1999). Animals can adapt to novel environmental conditions via various mechanisms, which operate on different timescales. Behavioural responses, such as changes in reproductive and survival-related behaviours, often operate on short timescales, ranging from seconds to days, and are for many organisms the first line of defence when dealing with rapid environmental change (Lowry et al., 2013; Sol et al., 2013; Wong and Candolin, 2015).

Many behavioural responses are proximately controlled by common underlying mechanisms, which include quick responses via the nervous system interacting with longer lasting responses through endocrine systems. Endocrine responses often include changes in circulating steroid hormones, such as glucocorticoids (e.g. cortisol and corticosterone) and sex hormones (e.g. androgens), to modulate a variety of behaviours, including behaviours related to reproduction and survival (reviewed in Emerson and Hess, 2001; Harris and Carr, 2016; Leary and Baugh, 2020; Sapolsky et al., 2000). Animals can alter their endocrine phenotypes in response to environmental changes and often rely on sensory cues to do so (Angelier and Wingfield, 2013; Halfwerk and Slabbekoorn, 2015). Rapid adaptation to changes to the sensory environment is therefore particularly likely to involve endocrine-related behavioural changes.

The sensory environment in urban areas drastically differs from

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natural areas, mainly due to the introduction of sensory pollutants such as anthropogenic noise (e.g. from traffic and construction) and artificial light at night (ALAN, e.g. street lighting) (Kyba et al., 2017; Votsi et al., 2017). Studies across a wide range of taxa have demonstrated endocrine responses, particularly in glucocorticoid and androgen levels, to exposure to anthropogenic noise (e.g. Kaiser et al., 2015; Mills et al., 2020; Tennessen et al., 2014) and ALAN (e.g. Dominoni et al., 2013; Forsburg et al., 2021; Grunst et al., 2020). Moreover, urban and forest populations can differ in their endocrine responses to sensory pollutants (Davies et al., 2017; Dominoni et al., 2021; Tennessen et al., 2018), pointing to population differences in endocrine plasticity (reviewed in Angelier and Wingfield, 2013; Bonier, 2023; Taff and Vitousek, 2016). While data on endocrine responses to sensory pollution are accumulating, general patterns and links with behaviour are often unclear (reviewed in Bonier, 2012, 2023; Injaian et al., 2020; Sinclair et al., 2022). Furthermore, understanding whether endocrine responses to sensory pollution are adaptive (i.e. increase fitness) requires species-specific knowledge on associated behaviours, and ultimately, fitness consequences in the context of urbanisation.

The túngara frog (*Engystomops pustulosus*) is a common species with reported behavioural divergence between urban and forest populations and associated fitness consequences (Halfwerk et al., 2019). Frogs in urban areas show higher sexual signalling effort, lower vigilance and a higher degree of behavioural flexibility, which appear to reflect adaptive responses to relaxed natural selection (from predators and parasites) and stronger sexual selection in urban areas (Halfwerk et al., 2019). Adaptive responses in urban areas appear to be partially driven by noise and light pollution (Cronin et al., 2022a; Smit et al., 2022). Whether endocrine responses proximately underlie the behavioural changes in response to urbanisation in the túngara frog is the focus of the current study.

Our aim was therefore to investigate endocrine phenotypes in urban and forest túngara frogs and evaluate any links to noise and light pollution. Using non-invasive techniques (see Baugh et al., 2018; Baugh and Gray-Gaillard, 2021) we quantified excreted hormone release rates in wild males from urban and forest environments, both in the field and in the lab, including before and after exposure to anthropogenic noise and/or ALAN. We focused on testosterone (T) and corticosterone (CORT), because these hormones are known to be related to sexual signalling and anti-predator behaviour in frogs (e.g. Mangiamele et al., 2016; Marler and Ryan, 1996; Narayan et al., 2013), and can interact in predicting risk-taking behaviour, with high T and low CORT being jointly associated with high risk-taking (Mehta et al., 2015).

We predicted that 1) urban frogs would have higher T and lower CORT levels compared to forest frogs in the field, given that risk-taking behaviour has been linked to higher T (reviewed in Tobiansky et al., 2018) and lower CORT levels (e.g. Baugh et al., 2017a; Baugh et al., 2017b) and urban túngara frogs exhibit more risky behaviour (i.e. higher call effort and lower vigilance) in the field (Halfwerk et al., 2019; Smit et al., 2022). Next, because we expected urban and forest environments to be associated with endocrine phenotypes, we predicted 2) differences between urban and forest endocrine phenotypes found in the field to be smaller or absent under lab conditions. Last, because sexual signalling in urban túngara frog has been shown to be more plastic (Halfwerk et al., 2019), we predicted that 3) urban frogs would have a higher degree of endocrine plasticity in response to anthropogenic noise and/or ALAN.

2. Methods

2.1. Sample collection

2.1.1. Frogs and sampling sites

We collected hormone samples from male túngara frogs (*Engystomops* (= *Physalaemus*) *pustulosus*), both directly in the field as well as after keeping the frogs under lab conditions and manipulating the sensory environment. Calling males were collected at urban sampling locations around Panama City and the canal zone, and in forest sites situated in Soberanía and Camino de Cruces national parks in the Republic of Panamá, with at least 1 km between sites (see Smit et al., 2022, Table A.1). Urban sites had higher urbanisation scores compared to forest sites (mean ± SD: urban: 2.2 ± 1.2 forest: −1.9 ± 0.4), calculated using the presence of paved surfaces and density of vegetation and buildings obtained from aerial images (1 km2 around sampling location) using *UrbanisationScore* software (Seress et al., 2014). Furthermore, urban sites were characterised by the presence of artificial light at night (mean ± SD: 1.98 ± 1.6 lx, lux meter HT309, HT Instruments, illumination level reading range 0.01–400 lx, average of measurements upwards and four cardinal directions, breast height) whereas forest sites were illuminated only by moon and star light (mean ± SD: 0.03 ± 0.01 lx). Noise levels largely overlapped between urban and forest sites (forest: 46.1–58.5 dBA, 49.8–64.6 dBC, urban: 43.3–55.1 dBA, 48.9–62.9 dBC, SPL meter, Voltcraft SL-100; two opposite directions for 10 s; fast, low).

We verified that all collected frogs were unique by either taking a toeclip sample (also for genetic analyses) or by comparing ventral pictures. For one frog that we did not toeclip or photograph, we assumed that this was not a recapture. We measured body size (Snout-Vent Length [SVL], mm) and mass (g) for all frogs.

2.1.2. Field samples

We took field samples from calling males from four urban and four forest sites spread over six different nights in September and October 2021 (Table A.1). On each sampling night we collected samples between 19:30–01:15 at both an urban and a forest site, and we counterbalanced visiting order. Túngara frogs produce calls consisting of a "whine" optionally followed by one or more "chucks", which results in increased call complexity and attractiveness (Ryan, 1985). To confirm earlier reported differences in calling behaviour between frog in urban versus forest sites (Halfwerk et al., 2019; Smit et al., 2022), we quantified the local calling behaviour at the collection sites. We did this by noting calling behaviour for 1 min for the same number of frogs as we would collect for hormone samples afterwards (n = 52). Per focal male, we counted the number of calls ("call rate"), the maximum number of chucks per call ("maximum call complexity") and the number of nearby calling conspecifics ("chorus size"). In total we took field hormone samples from 26 frogs in four urban populations and 26 frogs in four forest populations, with 4–10 frogs per site (Table A.1).

2.1.3. Lab samples

Additionally, we collected 80 male frogs at six urban and seven forest sites 1–4 h after sunset in September and October 2019 (see Smit et al., 2022, Table A.1). We transported the frogs in small plastic containers to our lab at Smithsonian Tropical Research Institute (STRI) in Gamboa. In the lab we put the frogs in one of eight custom-built recording boxes (36x25x58cm, LxWxH) on the same night as capture (n = 48), or one night after (n = 32). Within these recording boxes frogs were housed in a small enclosure (18 × 11.5 × 13cm, LxWxH) with a shelter and a small bowl (ø 8.5 × height 4.0 cm) with dechlorinated water to call from. Each night, we provided a standardised social environment to motivate the frogs to produce calls by playing artificial conspecific calls (chorus ~74 dB, single rival ~80 dB, Smit et al., 2022 for details) using Visaton FR8WP speakers (frequency response 100 Hz to 20 kHz (−10 dB), connected to Renkforce T21 amplifiers). Calling behaviour was recorded for another study (Smit et al., 2022), and here we only looked at whether the frogs called and how many calls per hour they produced.

We manipulated light levels in the recording boxes using white broad-spectrum LEDs (Nichia, NSPW500DS, peak ~460 nm) and noise levels using JBL clip 3 speakers (frequency response 120 Hz – 20 kHz (-6 dB)) at ~25 cm of the middle of the enclosure, connected to an iPhone or iPod (Apple). During daytime (07:00–19:00) we exposed frogs to ~250 lx to simulate day light levels. The first night in the recording boxes all

frogs were exposed to standardised lab conditions which entailed low night light levels (< 0.01 lx measured on the location of the frog, lux meter HT309, HT Instruments) and no noise playbacks. We took the lab samples the morning after (~8-12 h or ~32-36 h after capture from the field), starting between 10:30 and 11:45.

The second and third night, frogs were exposed to one out of four sensory treatments: forest (<0.01 lx) or urban (~1.3 lx) light levels during the night (measured at the position of the frog, lux meter HT309, HT Instruments), and a continuous forest or urban noise playback, which were synthesised based on field recordings and differed in peak amplitude (dBA: forest ~45, urban ~65; in the range reported by Halfwerk et al., 2019), amplitude modulation and frequency profile (for details Smit et al., 2022; Cronin et al., 2022a). We allocated frogs in a random but balanced manner regarding frog origin to the sensory treatments: "Control" (forest noise and forest light), "Light" (forest noise and urban light), "Noise" (urban noise and forest light) and "Noise + Light" (urban noise and urban light). We took the sensory treatment hormone samples again between 10:30 and 11:45 in the morning, which was after two nights of light treatment and ~40 h of the noise treatment.

2.1.4. Collecting hormone samples

We collected hormone samples at different time points after capturing frogs from the field (field samples: ~0 h, lab condition samples: ~12-36 h, sensory treatment samples: ~60-84 h). To obtain and quantify water-borne testosterone (T) and corticosterone (CORT) release rates, we used non-invasive methods previously validated in aquatic anurans (reviewed in Bastien et al., 2018; Narayan et al., 2019), including in túngara frogs (Baugh et al., 2018; Baugh and Gray-Gaillard, 2021). After briefly rinsing frogs in dechlorinated water using gloves, we placed individual frogs for 60 min in unused plastic cups containing 25 mL milliQ water with dissolved aquarium salts (Kent Marine; R/O Right, 0.7 g/L) with another plastic cup placed slight above the water level to prevent the frog from spending time out of the water. After collecting the samples, we immediately stored them in unused 50 mL tubes in a freezer (−7 °C, then −20 °C) for up to two months (field and lab condition samples) or two years (sensory treatment samples) until extraction.

3. Sample processing and analysis

3.1. Solid phase extraction

Following Baugh et al., 2018, we extracted water samples using solid phase columns (Sep-Pak C_{18}, 500 mg sorbent, Waters Corp., Milford, MA) attached to a vacuum manifold (Waters Corp., Milford, MA) and vacuum pump. We activated and equilibrated columns by pipetting 0.8 mL methanol (ACS grade) repeated five times, followed by five times 0.8 mL milliQ water. Next, thawed frog hormone samples were poured in the columns, processed slowly. After this, we washed the columns with 4 mL milliQ and let them run completely dry to avoid transporting cartridges loaded with methanol. After washing, loading and drying, we stored the columns in a freezer (−20 °C) until transportation at room temperature to Vrije Universiteit Amsterdam where we stored them again (−20 °C). The first processing steps and transportation to Amsterdam were carried out in October/November 2019 for the lab condition samples, and in November 2021 for the field and sensory treatment samples. Next, we eluted each sample using 3 mL methanol (HPLC grade) into borosilicate vials using the vacuum manifold. Samples were evaporated at 37 °C under a stream of nitrogen gas and frozen again (−20 °C) until reconstitution. We eluted and dried the lab condition samples in May 2020 and the field and sensory treatment samples in April and May 2022.

3.2. Liquid chromatography-mass spectrometry

We determined T and CORT release rates from our samples using liquid chromatography-mass spectrometry (LC-MS) based on methodology described by Evangelista et al., 2024. We reconstituted the field and sensory treatment samples in 500 μL methanol (HPLC grade). The lab conditions samples were dissolved in 250 μL of a mixture of 95 % assay buffer (enzyme immunoassay kits) and 5 % ethanol (95 %), (for details see Fig. S1). For both T and CORT, we added 50 μL stable isotope labelled internal standards to each sample to be able to correct recovery rates (mean ± SE: lab condition samples: T: 96.1 ± 1.4 %, CORT: 70.6 ± 1.5 %, field and sensory treatment samples: T: 77.6 ± 1.4 %, CORT: 79.3 ± 2.2 %). We filtered the field and sensory treatment samples using 0.2 μm pore polypropylene filters (Agilent Captiva, Agilent Technologies, Santa Clara, CA, USA), and dried them at 40 °C (CentriVap® Refrigerated Vacuum Concentrators, Labconco®, Kansas City, MO). Next, we resuspended the field and sensory treatment samples in 75 μL of methanol/water (MeOH:H_2O = 1:1, HPLC grade). Additionally, to make a standard curve we prepared 50 μL serial dilutions from stock solutions (T: 9 dilutions from 8.853 to 0.446 pg, CORT: 10 dilutions from 9.635 to 0.226 pg) to which we added 50 μL of the internal standard and 100 μL milliQ water. Finally, we ran the LC-MS (SCIEX Triple Quad™ 6500+ mass spectrometer, SCIEX Exion LC system) by running the samples through columns (C_{18}, 2.6 μm, 100 × 2.1 mm Kinetex®, Phenomenex) with injection volume of 10 μL using a flow rate of 0.6 mL/min and a solvent gradient of MilliQ water (A) with 0.2 mM% NH4F and methanol absolute (B).

We estimated T and CORT release rates from the standard curve while correcting for individual recovery rates. We determined technical repeatability by remeasuring (two or three times) six samples within the linear range of the standard curve and calculating the average CV% between runs to be 7.0 % for T and 7.5 % for CORT. Using LC-MS we were unable to estimate values for four out of 206 T samples and for 29 out of 206 CORT samples. For samples with values below (lab conditions samples: T: forest frogs: $n = 7$, urban frogs: $n = 15$, CORT: urban frogs: $n = 5$, sensory treatment samples: CORT: urban frogs: n = 1) and above (lab conditions samples: CORT: forest frogs: n = 1) the linear range of the standard curve we accepted the possibly less accurate values. We decided to do include all samples, since most samples (75 %) below the standard curve were obtained from urban frogs and excluding these low values would therefore bias the results. Additionally, we applied enzyme immunoassays (EIA), another commonly used technique for determining hormone levels, to the lab conditions samples to compare with the results from the LC-MS (for details see Fig. A.1).

3.3. Correlations and sample sizes

We corrected for variation in sample volume (7.5–36.5 μL) for the lab conditions samples for the LC-MS, since these samples were also used for the EIA. Next, we corrected all values for frog size for by dividing by body size (SVL, mm), providing us with release rates in (pg/SVL_{mm}/h). Finally, we obtained hormone release rates using LC-MS (range: T: 0.06–4.0, CORT: 0.06–19.4 pg/SVL_{mm}/h, mean ± SD: T: 1.0 ± 0.8, CORT: 2.2 ± 2.7 pg/SVL_{mm}/h) and EIA (range: T: 1.7–227.5, CORT: 0.4–29.5 pg/SVL_{mm}/h, mean ± SD: T: 22.9 ± 32.4, CORT: 5.8 ± 6.1 pg/SVL_{mm}/h), which were strongly correlated (Spearman correlation, T: $\rho = 0.78$, $p < 0.001$, $n = 78$, CORT: $\rho = 0.84$, $p < 0.001$, $n = 68$, Fig. S1). Because of higher sensitivity of the LC-MS, we only report the results from LC-MS in the main text. Analysis of EIA data showed similar patterns, which we report in the supplementary materials (Table A.3,4,6). Finally, we obtained well-balanced sample sizes between the urban and forest frogs as well as between the different sensory treatments for the LC-MS samples, see Table 1.

3.4. Statistical analysis

We used R (v. 4.2.2, Team R Development Core, 2022) to run binomial (logit link), gaussian (identity link) and (generalised) Poisson (log link) linear mixed models (GLMM) using the *lme4* (v. 1.1-31, Bates et al., 2015) and *glmmTMB* packages (v. 1.1.5, Brooks Mollie et al.,

Table 1
Sample sizes for testosterone (T) and corticosterone (CORT) obtained using LC-MS for urban and forest frogs for samples obtained in the field, under lab conditions and after sensory treatments.

	Urban frogs		Forest frogs	
	Testosterone	*Corticosterone*	*Testosterone*	*Corticosterone*
Field	25	24	26	25
Lab conditions	39	39	40	39
Sensory treatments				
• Control	8	7	10	9
• Noise	10	7	10	9
• Light	10	8	10	9
• Noise + Light	10	7	10	8

2017). Hormone release rates (T and CORT) were $\log_{10}$-transformed to improve normality of model residuals. Numerical covariates were standardised by calculating z-scores. Absence of heteroskedasticity was verified by visual inspection of the fitted values plotted against the residuals, and dispersion was checked using the *DHARMa* package (v. 0.4.6, Hartig, 2021). We tested for significance by comparing the model with and without the factor of interest using likelihood ratio tests (Zuur and Ieno, 2016) and visualised raw data using *ggplot2* (v. 3.4.1, Wickham, 2009). All linear mixed models contained 'site ID' as a random intercept to account for multiple values per collection site as well as frog origin (urban/forest) as fixed effect.

To test whether local calling behaviour differed between urban and forest sites, we ran Poisson models with call rate and maximum call complexity as response variables. All models on local calling behaviour contained 'chorus size' as covariate, as this known to influence calling behaviour. Since models with Poisson distributions indicated under- or overdispersion, we used generalised Poisson distributions instead. To investigate the association between calling behaviour in the lab and hormone release rates (T and CORT) the morning after, we ran binomial models including whether the frog called (yes/no) and gaussian models with the number of calls per hour as response variables. Additionally, the models on lab conditions samples always contained 'sampling moment' (after 12/36 h), while the models on sensory treatment samples contained the interaction between 'noise treatment' (urban/forest) and 'light treatment' (urban/forest). We tested for correlations between T and CORT release rates across all data sets for urban and forest frogs separately as well as on the origins combined by running spearman's rank correlation tests.

To test for the effect of frog origin on hormone release rates in the field as well as in the lab, we tested the fixed effect 'frog origin' (urban/forest). We investigated the effects of the sensory treatments by running analyses on the urban and forest frogs separately. We started with testing the interaction between 'noise treatment' and 'light treatment'. A significant interaction was followed up by a comparison of the observed combined effects (sum of intercept, observed single effects and interaction effect) versus expected additive effects (sum of intercept and observed single effects), both obtained from the model including the interaction term. In this way we characterised interactions as 'synergistic' or 'antagonistic' (following Hale et al., 2017; Halfwerk and Jerem, 2021). Next, we removed the interaction and tested for significance of the main effects of noise and light treatment separately. The reported estimates for main effects of noise and light treatments were obtained from the models excluding the interaction term. Estimates reported in the main text were back transformed from the logarithmic scale. See supplementary Tables S2–8 for a complete overview of the results.

4. Ethical note

Experiments were licensed and approved by STRI (IACUC permit: 2019-0301-2022) and the Autoridad Nacional del Ambiente de Panamá (SE/A-47–19 and SE/A-31-2020). Frogs kept in the lab were fed wild caught termites on the third or fourth day in the lab. We released the frogs at their capture sites, either immediately after taking a hormone sample in the field or after four to six nights in the lab.

5. Results

5.1. Calling behaviour in the field and in the lab

Frogs in urban areas had higher (+45.5 %) call rates (GLMM, $n = 52$, $\chi2 = 4.09$, $p = 0.04$, Table A.2) and higher (+64.9 %), but not statistically significantly different, maximum call complexity ($\chi2 = 1.44$, $p = 0.23$) compared to frogs in forest areas. For the proportion of frogs that called in the lab (night 1: 30/80, night 3: 36/80), we were able to obtain both testosterone (T) and corticosterone (CORT) release rates for a subset (night 1: $n = 25$, night 3: $n = 34$). In general, we found hormone levels to be unrelated to calling behaviour in the lab (all $p > 0.08$, Table A.3), except for the fact that frogs producing fewer calls under lab conditions (night 1) had higher CORT levels the morning after ($\chi2 = 6.28$, $p = 0.01$). Last, we found T and CORT release rates to be uncorrelated in all data sets, both for urban and forest frogs combined as well as within each origin (all $p > 0.09$, Table A.4).

5.2. Urban and forest endocrine phenotypes vary in the field and in the lab

When directly measured in the field, T levels did not vary between urban and forest frogs (GLMM, $n = 51$, $\chi2 = 0.70$, $p = 0.40$, Fig. 1A, Table A.5), but urban frogs had lower (−44.9 %) CORT levels compared to forest frogs ($n = 49$, $\chi2 = 5.33$, $p = 0.02$, Fig. 1B). When keeping frogs under standardised lab conditions, urban and forest endocrine phenotypes again differed (Table A.6). Both T (−36.7 %) and CORT levels (−44.5 %) were found to be lower in urban frogs compared to forest frogs (T: $n = 79$, $\chi2 = 6.09$, $p = 0.01$, CORT: $n = 78$, $\chi2 = 5.06$, $p = 0.02$, Fig. 1C—D). During the sensory treatments, urban frogs still had overall lower (−43.6 %) T levels compared to forest frogs (n = 78, $\chi2 = 7.24$, $p = 0.007$, Table A.7), whereas CORT levels did not vary anymore with frog origin ($n = 64$, $\chi2 = 0.03$, $p = 0.86$).

5.3. Noise and light pollution affect urban and forest endocrine phenotypes differently

We exposed frogs to urban or forest noise and light levels in our lab set-up and tested their T and CORT levels responses to the sensory treatments. Forest frogs did not change their T or CORT levels in response to any of the sensory treatments (GLMM, T: $n = 40$, CORT: $n = 35$, all $p > 0.07$, Fig. 2C—D, Table A.8). In contrast to the forest frogs, urban frogs responded to the sensory environment by adjusting both their T and CORT levels (T: $n = 38$, CORT: $n = 29$, Fig. 2A-B, Table A.8).

For urban frogs, exposure to urban light levels lead to a strong increase in T levels (+86.0 %) compared to forest light levels ($\chi2 = 4.22$, $p = 0.04$, Fig. 2A). Noise levels nor the interaction between noise and light treatment affected T levels in urban frogs (all $p > 0.59$). In terms of CORT levels, we found an interaction effect between urban noise and light exposure on CORT level in the urban frogs ($\chi2 = 11.07$, $p < 0.001$, Fig. 2B). Compared to the forest noise and light treatment ('control'), urban frogs had lower (−47.2 %) CORT levels when exposed to a combination of noise and light pollution. The observed interaction effect is less negative than the expected effect under additivity of exposure to the separate pollutants (−92.2 %), and therefore this interaction can be classified as an antagonistic effect, possibly indicating that urban frogs could not further lower their CORT levels ('floor effect'). Furthermore, we found a main effect of urban noise on CORT in urban frogs ($\chi2 = 3.89$, $p = 0.049$). Urban frogs lowered their CORT levels (−47.1 %) in response to urban noise compared to forest noise exposure. Exposure to urban light alone did not affect CORT release rates in urban frogs ($\chi2 <$

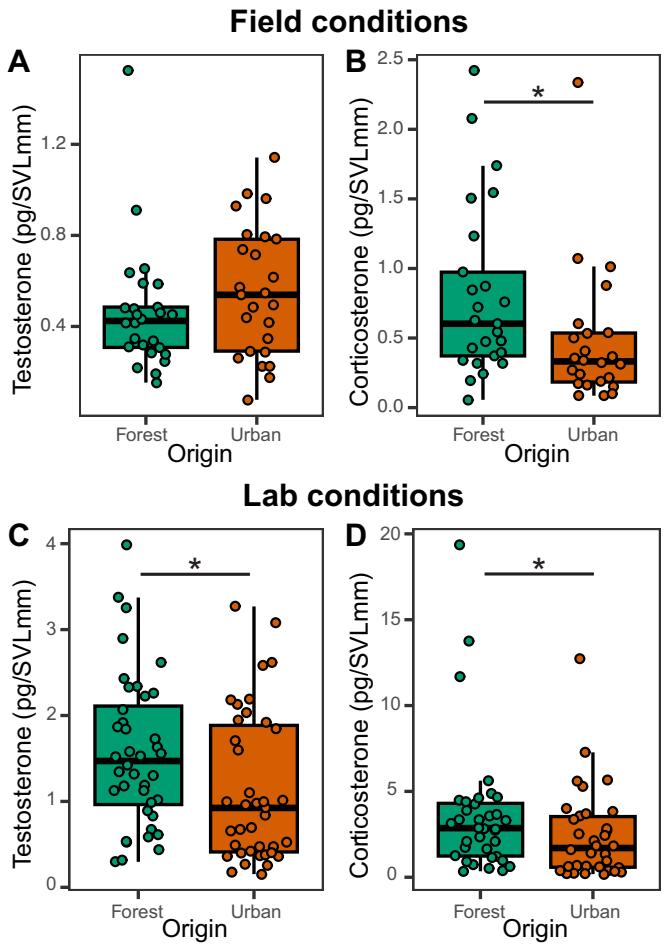

Fig. 1. Testosterone (T) and corticosterone (CORT) release rates (pg/SVL_{mm}/h) in urban and forest frogs in the field (A, B) and after 12-36 hours under standardised lab conditions (C, D). Statistically significant differences are indicated with an asterisk (* < 0.05). Graphs depict untransformed hormone release rates. See text and Table A.5-6 for statistics.

0.01, $p = 1.00$).

6. Discussion

We tested the hypothesis that two steroid hormones, testosterone (T) and corticosterone (CORT), are associated with divergent behavioural phenotypes among male urban and forest túngara frogs, and that exposure to anthropogenic noise and/or artificial light at night (ALAN) act as a driver. We quantified T and CORT release rates in the field and in the lab before and after exposure to urban noise and/or light. Non-invasive water borne hormone sampling, measuring hormones excreted via the urine, skin, or mucus, reflects the endocrine state of individuals over a relatively long window of time, and allowed us to repeatedly measure hormones and behaviour (Baugh et al., 2018; Baugh and Gray-Gaillard, 2021). We show that urban and forest frogs differ in their endocrine phenotypes in the field as well as under lab conditions, and that only urban frogs show plastic endocrine responses to sensory pollution exposure.

6.1. Impacts of urbanisation on endocrine phenotypes

When sampling calling frogs in urban and forest areas, we found that CORT but not T was different between the urban and forest populations. Urban frogs had lower CORT levels, which was in line with our predictions and observation that túngara frogs in urban areas invest more in sexual signalling and less in anti-predator behaviours (also see Cronin et al., 2022a; Halfwerk et al., 2019; Smit et al., 2022). The field samples were taken at night when the frogs were already calling, and the observed release rates therefore likely include the hormonal states of the individuals during daytime (i.e. before they started calling). With our field data we add to a growing body of observational field studies into endocrine differences between urban and forest populations, but general patterns between CORT levels and urbanisation across studies, however, are difficult to interpret (Bonier, 2012; Iglesias-Carrasco et al., 2020; Injaian et al., 2020), owing to the highly species specific effects of urbanisation on diet, physiology, stressors and behaviour, and correlational nature of many of the earlier studies. Therefore, understanding the effects of urbanisation requires experimental manipulations, such as standardisation of the environment and manipulation of urban factors, to assess causal relationships.

To assess population of origin differences, we kept urban and forest frogs under standardised lab conditions, which included playbacks of conspecific calls. Because we obtained lab condition samples in the morning, the hormone release rates might reflect endocrine states during the night prior to sampling, although some carry-over effects from the field are possible. Therefore, circumspection is necessary when comparing lab and field collected samples, since samples were taken at different times of day (lab samples at morning, field samples at night), as differences could be caused by diel rhythms or the switch from field to lab conditions, or both. Because we expected that endocrine phenotypes would be associated with urban and forest environments, we predicted that differences between populations of origin would be smaller or absent under lab conditions. Contrary to our prediction, we found that under standardised lab conditions, CORT levels in urban frogs were again lower compared to forest frogs, but that this difference was absent during the sensory treatments. Additionally, in the lab the forest frogs showed higher T levels compared to urban frogs, both before and after the sensory treatments, a population difference we did not find in the field samples. Lab conditions, including the conspecifics playbacks, might have affected urban and forest endocrine phenotypes differently because of the differential change in the environment for frogs from different origins. For example, forest frogs heard lower call rates and call complexity in the field prior to collection compared to urban frogs (also see Halfwerk et al., 2019; Smit et al., 2022) and the conspecific playbacks may thus be perceived as an increase in the competitive environment for the forest frogs. Male túngara frogs are behaviourally sensitive to temporal changes in the complexity of rivals' calls (Baugh and Ryan, 2010; Bernal et al., 2009), and the sounds of calling rivals can lead to an increase in T, as has been reported in American green tree frogs (*Hyla cinerea*) (Burmeister and Wilczynski, 2000; but see Still et al., 2019). Therefore, the relative increase in the vocal-competitive environment induced by conspecific playbacks might have activated the hypothalamic-pituitary-gonadal (HPG) axis resulting in higher T levels in forest compared to urban frogs in the lab. Overall, differences between urban and forest endocrine phenotypes under lab conditions indicate that, besides direct environmental effects, there are also other, longer lasting, factors shaping the urban and forest frogs' endocrine phenotypes.

Last, we exposed urban and forest frogs to urban noise and/or light to assess their endocrine response to sensory pollution. Forest frogs did not alter their hormone levels according to sensory treatments, whereas urban frogs increased their T in response to urban light and decreased their CORT in response to urban noise, which was in line with our predictions. Additionally, we show an interaction effect between urban light and noise on CORT levels in urban frogs. Urban frogs lowered their CORT levels in response to combined urban noise and light less than expected under additivity of exposure to the separate pollutants, which points towards a floor effect (Halfwerk and Jerem, 2021). Whether urban and forest populations differ in their endocrine responses to urban sensory pollution has only been assessed in a handful of studies, which have focused on one sensory pollutant at the time (Bonier, 2023;

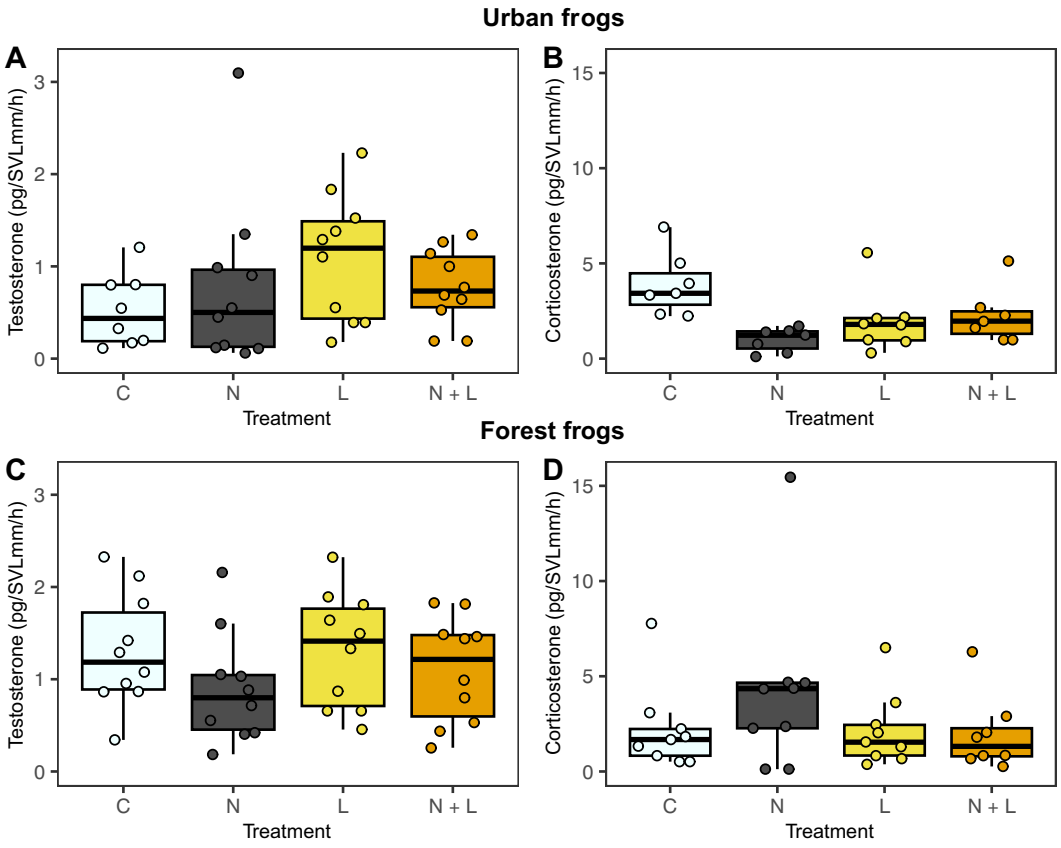

Fig 2. Testosterone (T) and corticosterone (CORT) release rates (pg/SVL_{mm}/h) in response to exposure to sensory treatments (C: forest noise and forest light, N: urban noise and forest light, L: forest noise and urban light, N + L; urban noise and urban light) for urban (A, B) and forest frogs (C, D). Graphs depict untransformed hormone release rates. See text and Table A.6-7 for statistics.

Seebacher, 2022). While our results indicate that urban but not forest frogs adjust their hormone levels to sensory pollutants, earlier studies have reported dampened CORT responses to traffic noise in urban versus forest wild house wrens (*Troglodytes aedon*) and in lab-reared wood frogs (*Rana sylvatica*) originating from noisy versus quiet sites (Davies et al., 2017; Tennessen et al., 2018). On the other hand, ALAN exposure of blue tit (*Cyanistes caeruleus*) nestlings led to an increase in feather CORT in urban chicks, while the reverse pattern was visible in forest chicks (Dominoni et al., 2021). It has been proposed that expressing an adaptive endocrine phenotype in response to sensory pollution might be facilitated by a higher degree of endocrine plasticity (reviewed in Angelier and Wingfield, 2013; Bonier, 2023; Taff and Vitousek, 2016), and our results support this idea. Nevertheless, endocrine responses in urban versus forest populations still needs to be assessed across more species and, most importantly, linked to associated behaviours and fitness consequences in the context of urbanisation.

6.2. *Adaptive or maladaptive responses to urbanisation*

Understanding whether changes in endocrine phenotypes represent adaptive (either genetic or plastic) responses requires knowledge of associated behaviours and their fitness consequences. Hormones such as T and CORT, proximally underlie many aspects of behaviour, including fitness-related behaviours linked to reproduction and survival (reviewed in Emerson and Hess, 2001; Harris and Carr, 2016; Leary and Baugh, 2020). Specifically CORT levels are known be modulated by environmental cues (Sapolsky et al., 2000), and higher CORT levels in frogs might correspond to decreased investment in sexual signalling (Emerson and Hess, 2001; Leary et al., 2006; Marler and Ryan, 1996, but see Still et al., 2019) and increased investment in anti-predator behaviours (Harris and Carr, 2016; Narayan et al., 2013). In our study, we show a negative association between the number of calls produced per hour in the lab and CORT levels the morning after, but no associations between calling behaviour and T levels. Additionally, CORT and T levels have been proposed to balance each other and underlie transitions between, for example, producing sexual signals and restoring reserves in frogs (Emerson and Hess, 2001). We did not find any correlations between these hormones, neither within urban and forest origins, nor when the populations were pooled, but an earlier study on túngara frogs found a positive association between CORT and T, illustrating the need for further research (Still et al., 2019).

Urbanisation can induce behavioural changes in animals (Lowry et al., 2013; Sol et al., 2013; Wong and Candolin, 2015), particularly via changes in the sensory environment (Dominoni et al., 2020; Halfwerk and Slabbekoorn, 2015). Sexual traits, of central importance for determining fitness, have shown to be affected by sensory pollutants, including anthropogenic noise and ALAN, but these changes have not often been linked to underlying physiological mechanisms (Cronin et al., 2022b). In our study, we showed that under urban noise and light exposure urban túngara frogs increased their T levels and decreased their CORT levels compared to forest conditions, which is in line with behavioural data that showed increased calling effort and decreased vigilance in urban areas (Halfwerk et al., 2019), and specifically under sensory pollution exposure (Cronin et al., 2022a; Smit et al., 2022). Interestingly, we found that urban noise and light did not elicit endocrine responses in the forest frogs, suggesting that these sensory pollutants do not always directly impact endocrinology and associated behaviours. The behavioural and endocrine changes in urban túngara frogs are on the other hand presumably adaptive since there are fewer attracted predators, parasites and females in urban areas, and thus increasing sexual signalling and decreasing vigilance in response to sensory pollution would ultimately lead to higher reproduction, without

paying any increased costs to survival (Halfwerk et al., 2019).

In our study we shed light on the endocrine responses in sexual signal producers, but understanding how anthropogenic activities affect receiver responses to sexual signals is essential for determining fitness consequences (Candolin and Wong, 2019). Few studies, however, have assessed how receivers change their endocrine phenotypes in response to sensory pollution, even though hormones such as CORT are known to affect mate choice in frogs (e.g. Baugh et al., 2021; reviewed in Leary and Baugh, 2020). Female wood frogs (*Rana sylvatica*), for example, increase their CORT and decrease their attraction to male sexual signals when exposed to traffic noise (Tennessen et al., 2014), which could be caused by signal masking or distraction (Dominoni et al., 2020). In túngara frogs, fewer females are attracted to playbacks of frog calls in urban areas, but it is unclear how and why this pattern arises (Halfwerk et al., 2019). It would be interesting to assess whether females from urban and forest areas also have divergent endocrine responses to urban sensory pollutants, which would provide more insights in the effects of urbanisation on inter-sexual selection.

6.3. Plasticity versus genetics in differential endocrine phenotypes

Our results show that endocrine phenotypes differed between urban and forest frogs in the field as well as after a few days in the lab, suggesting that direct environmental effects alone do not explain the differences between urban and forest frogs. Moreover, urban frogs adjusted their endocrine phenotypes to both urban light and noise exposure, whereas forest frogs did not alter their hormone levels in response to sensory pollution. How an individual expresses its endocrine phenotype under different environmental conditions is shaped by both their baseline hormone levels and their perception and/or processing of environmental cues (reviewed in Bonier, 2023; Halfwerk and Slabbekoorn, 2015; Taff and Vitousek, 2016).

In the current study, the hormone estimates likely reflect baseline levels, though it is possible that the experience of water bath collection and transport to the lab acted as stressors (Dickens et al., 2009). However, even if that was the case, it is important to note that such handling and transport methods have been used for decades in túngara frog behavioural research, and yet robust sexual behaviour is typically elicited from most individuals. Since baseline hormone levels only reflect one aspect of an individual's endocrine phenotype, future work should focus on probing the plasticity of the hypothalamic-pituitary-interrenal (HPI) axis by estimating, for example, the scope of secretory reactivity in response to stressors as well as the recovery dynamics and their upstream receptor underpinnings, which are known to be important for understanding risk-taking behaviour in birds (e.g. Baugh et al., 2017a, 2017b). Common toad (*Bufo bufo*) tadpoles from urban and agricultural sites, for example, show a stronger negative glucocorticoid feedback after a stressor compared to tadpoles from natural habitats (Bókony et al., 2021).

Population variation in endocrine phenotypes can be a result of environmental variation and heritable genetic variation. Within an individual's lifetime their endocrine phenotypes can be modulated via phenotypic plasticity (West-Eberhard, 1989), including recent experience ('habituation' or 'sensitisation') and the developmental environment ('developmental plasticity'). Exposure to anthropogenic noise and ALAN during development has indeed been shown to affect endocrine phenotypes (e.g. Dominoni et al., 2021; Grunst et al., 2020; Tennessen et al., 2018). Additionally, genetic differences accumulated over generations, due to either drift or adaptive evolution, can underlie individual variation in endocrine phenotypes (Béziers et al., 2019; Evans et al., 2006; Jenkins et al., 2014; Stedman et al., 2017). There are some experimental studies suggesting heritable differences in CORT responses between urban and non-urban populations, including in dark-eyed juncos (*Junco hyemalis*), house wrens (*Troglodytes aedon*) and blackbirds (*Turdus merula*), although early developmental effects (e.g. maternal effects) could not be ruled out (Atwell et al., 2012; Ouyang et al., 2019; Partecke et al., 2006). In our study, the frogs from urban areas most likely had more experience with sensory pollution, both recently and during their development, compared to the forest frogs. Plasticity is therefore the most likely mechanism to have contributed to an increased responsiveness to urban noise and light exposure in urban frogs, but genetic processes underlying endocrine response differences cannot be ruled out. Conclusive experiments on underlying mechanisms of urban and forest population differentiation in their endocrine phenotypes, specifically in response to urban factors, are required to assess the potential for urban evolution and speciation (Bonier, 2023; Cronin et al., 2022b; Halfwerk, 2021; Lambert et al., 2020).

7. Conclusion

In conclusion, we show that male urban and forest túngara frogs exhibit different endocrine phenotypes in the field as well as in the lab. Moreover, urban frogs showed endocrine responses to anthropogenic noise and ALAN, whereas forest frogs did not alter their hormone levels in response to sensory pollution. Stronger endocrine responses could be related to increased behavioural flexibility, allowing animals to cope with challenges brought along by urbanisation. In our study system the reported differences in endocrine phenotypes likely proximally underlie behavioural divergence between urban and forest frogs, which is presumably an adaptive response to changed natural and sexual selection pressures due to urbanisation. Our study provides insights in how urbanisation, and specifically anthropogenic noise and ALAN, affect endocrine phenotypes underlying important behavioural traits.

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Data availability

Data and R scripts are available at DataverseNL, and can be accessed via doi:10.34894/TA0XUT.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2023.105453.

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