Abstract

1. If environments stay relatively constant over an individual's lifetime, a juvenile that accurately perceives environmental conditions, like population density, may adjust adult traits to better match their environment, thereby increasing success. While previous studies have explored how adult exposure to population density affects physiological and behavioural plasticity, the influence of juvenile density experience on adult traits is less studied.
2. Using the common house cricket, *Acheta domesticus*, we explored whether perceived acoustic population density during development affected adult physiology and behaviour. We simulated high- and low-densities using live ambient male song. Upon maturation, we measured metabolic (resting respiration) rate, reproductive investment (testes and accessory gland masses), calling song characteristics and aggressive behaviours from pairwise contests between males from different densities.

KEYWORDS
aggressive behaviour, call characteristics, developmental plasticity, mating tactics, physiology, population density

INTRODUCTION

Reproductive behaviour, morphology and physiology can vary discontinuously among individuals of a species (i.e., alternative reproductive phenotypes; Brockmann, 2001; Gross, 1996; Oliveira et al., 2008; Shuster & Wade, 2003), which has long interested evolutionary biologists. This largely stems from the desire to explain the incredible diversity of such tactics found across animal species (Gross, 1996; Shuster, 2010). Alternative reproductive morphs can be influenced by environmental cues that are internal (e.g., body condition, size and age) or external—including abiotic (e.g., light, season and temperature) and biotic factors (e.g., social group, predation and population density; Brockmann, 2001; Gross, 1996). While the effects of internal environmental conditions (Brockmann, 2002; Forsyth & Montgomerie, 1987; Painting & Holwell, 2014) and abiotic external environmental conditions (Corlatti et al., 2020; Cusano et al., 2016; Denoël & Doellen, 2010) on tactics have been relatively well studied, biotic external environmental conditions have been much less studied (but see Formica & Tuttle, 2009; Ribeiro et al., 2010; Wilgers et al., 2014).

Population density—an external biotic environmental condition—has been shown to affect a variety of traits across taxa, such as reproductive success (Tveraa et al., 2013), early development (Albecker et al., 2020; Clutton-Brock et al., 1987), growth rate (Iba et al., 1995; Zewe & Booth, 2014), activity (Cade & Cade, 1992; de Rivera et al., 2003), acoustic signalling (Cade & Cade, 1992; Cade & Wyatt, 1984), female choosiness (Atwell & Wagner Jr., 2014; Holveck et al., 2015) and aggression (DiRienzo et al., 2012; Holveck et al., 2015; Knell, 2009). While many studies have found that adult environmental conditions can impact behaviours (Cade & Cade, 1992; Holveck et al., 2015), environmental conditions during development are known to have profound effects on adult behaviour in some systems (see Beach & Jaynes, 1954; Carlson, 2017).

Juvenile experience can influence behavioural and physiological responses upon emergence into adulthood due to delicate hormonal balances in the endocrine system associated with developmental moults (Dufty Jr. et al., 2002). Therefore, individuals may adaptively modify behaviour in response to environmental conditions experienced during development (Carlson, 2017). However, these behavioural changes are dependent on environmental conditions because a phenotype that may increase reproductive fitness in one environment could be maladaptive in another (Dufty Jr. et al., 2002).

Specifically, variation in population densities during development could entail physiological (e.g., metabolism or reproductive investment) or behavioural (e.g., aggression or mating behaviour) changes. Individuals with higher metabolic rates may be capable of performing longer or more frequent energy-demanding tasks, such as mate searching, organ development or predator/competitor interactions (Lorenz & Gäde, 2009). Both male weaponry and sperm production require energetic expenditure, leading to a trade-off between precopulatory and post-copulatory investments, respectively (Simmons & Emlen, 2006). When population densities are high, polyandrous species may experience heightened sperm competition (Buzatto et al., 2015), which may lead to increased investment in testes size (Vahed et al., 2011). High population densities also increase the probability of interactions between conspecifics competing for mates and resources, which can escalate to aggressive contests, which may be energetically costly or increase the risk of injury (Knell, 2009).

Density may also affect investment in mate advertisement. For example, in species where males use song to attract females, longer call duration may be more advantageous in lower densities due to the possible infrequency of mate interactions, while shorter call duration helps reduce song overlapping at high densities (Eiriksson, 1992). However, call durations might increase at higher densities if females prefer longer calls (Wells & Taigen, 1986). While no study to our knowledge has investigated the role of population density in song quality variation (i.e., by changing characteristics), we might expect high-density males to invest more in producing an attractive song (a song preferred by females) to stand out from the crowd.

The effects of population density on these factors remain understudied or show mixed results for different species. Therefore, we chose to examine how perceived population density during juvenile development affects adult metabolism, reproductive investment, calling song characteristics and aggressive behaviours in individual males using a cricket model system. Measuring all the traits in the same individuals allowed us to look for relationships among traits within individuals; as this is difficult to do in many systems, we believe this is a strength of this experimental design.

Study system

We decided to test the effects of juvenile population density on the above adult traits using commercially bred *Acheta domesticus*, the common house cricket. In the field, cricket population densities vary due to variations in resource distributions and environmental conditions (Weidemann et al., 1990). Little is known about physiological changes in crickets that experience different population densities. One study using *Teleogryllus oceanicus* found that male crickets in higher population densities invest more in reproductive tissue due to heightened sperm competition (Bailey et al., 2010). No study to our knowledge has investigated population density effects on cricket metabolism.

Crickets produce a calling song that varies in duration and call characteristics between individuals. Previous studies show that adult *Gryllus* field cricket males called longer at low population densities (Cade & Cade, 1992; Cade & Wyatt, 1984; French & Cade, 1989; Hissmann, 1990), indicating that population density may influence behaviours when calling for a mate. While we know that call duration is negatively related to density, it is largely unknown how population density influences the calling characteristics of male crickets. Chirp intensity (loudness), chirp rate, chirp duration, the number of pulses per chirp, pulse duration, dominant frequency and pulse period have all been found to be important parameters for female preference in various cricket species (Stout et al., 1983; Stout & McGhee, 1988). We do know that females become less discriminatory of call characteristics of *T. oceanicus* when reared in isolation (Bailey & Zuk, 2008).

There is some conflict in the literature as to what role population density plays on aggression. Species differ in patterns of aggression associated with increasing population density: some increase in aggression and others decrease (Knell, 2009). Opposite to studies using non-cricket organisms (Erwin & Erwin, 1976; Moore, 1987; Parker & Nilon, 2008), field crickets are often most territorial at lower densities (Alexander, 1961). In particular, *Gryllus bimaculatus* (Simmons, 1986—density manipulated for adults; Iba et al., 1995—density manipulated for juveniles by changing the number of individuals in containers) and *Gryllus integer* (DiRienzo et al., 2012—perceived density manipulated for juveniles by the playing of recorded song) showed a reduction in both the number and intensity of aggressive contests at higher densities. Also, previous studies have shown mixed effects of population density on the number of aggressive song bouts (Adamo & Hoy, 1995; DiRienzo et al., 2012). No study, to our knowledge, has investigated the role of perceived population density via live ambient song on reproductive investment, metabolism, calling behaviour and aggression for a single species, nor with measuring these multiple traits in the same individuals.

A. domesticus males produce three distinct songs: the calling song to attract a female from afar, the courtship song performed once the female approaches the male and an aggressive song towards a competing conspecific male (Gray, 1997). Male *A. domesticus* will produce any of the three song types in laboratory settings, but the aggressive and courtship songs require contact (either physical or substrate chemical cues; Assis et al., 2016) to initiate. A range of aggressive behaviours performed by *A. domesticus* can be observed and easily identified (Hack, 1997a). Although it is unclear whether *A. domesticus* will defend burrows in the wild, they will defend burrows and use them as calling sites in the lab (BP personal observation; Hack, 1997b).

In this study, we investigated respiration rates, reproductive investment, calling song characteristics and male–male aggression for adult *A. domesticus* reared in perceived high or low population density. We simulated population density using live ambient cricket song, without direct contact or chemical cues with conspecifics—a novel method to the best of our knowledge. This method has several advantages: individuals in different conditions do not encounter differing competition for food and do not have differing prior experience of aggressive interactions. Lastly, using live song as opposed to recorded songs means that singing occurs at biologically relevant times (e.g., at night) and naturally varies in intensity.

Low population densities may lead to higher resting metabolic rates based on previous findings that low densities experience heightened aggression and longer calling durations. However, if males in high densities invest more in reproductive organs, producing attractive songs or searching for mates, they may require higher metabolic rates or suggest an energetic trade-off if no differences are found between densities. In addition, we expected that males perceiving high-density environments would have larger testes and accessory glands (that produce seminal fluid) due to increased sperm competition. Aggressively guarding a territory may be too costly for males in higher densities due to increased risk of injury and energy expenditure associated with frequent male–male interactions. As a result, we predicted that males reared in a perceived higher density would invest more energy in mate attraction by producing a song with preferred characteristics and invest less in aggression to protect territory.

MATERIALS AND METHODS

General methods

For these experiments, we obtained commercially available common house crickets, *A. domesticus*, from Flukers Farms in June 2017. Until maturation, *A. domesticus* nymphs go through a series of eight instars and moults (Clifford et al., 1977). Previous studies show that nymphs are most susceptible to environmental influences on behaviour and physiology during the last two instars of their juvenile development (Friberg et al., 2011; Zera & Tiebel, 1989). Thus, we reared *A. domesticus* nymphs in the two (high or low) population density conditions from the start of their penultimate instar. We individually placed male crickets in their penultimate moult into small, clear plastic containers ($17 \times 10 \times 11$ cm). All containers had a paper towel substrate, cardboard egg crate shelter, ad libitum cat chow (Purina) and a water vial plugged with cotton. We placed all animals in Darwin Insect Rearing Chambers (IN055-AA-LT) maintained at 28°C, in a reversed light:dark cycle (LD 14:10 h). Nymphs went through multiple moults and several weeks under these reversed conditions. We monitored temperature and humidity in the chambers throughout the experiments using HOBO ProV2 U23-001 loggers, collecting measurements every 30 min.

We used live ambient male cricket song to simulate high- and low-density conditions. We placed nymphs in the high-density treatment in a rearing chamber with approximately 20–30 adult *A. domesticus* males, who call in the absence of female cues. These non-test males were housed in individual containers, as described above for the test crickets, and were placed on the shelf below our test animals (less than 30 cm between the test and non-test males). Adult *Gryllus campestris* males produce calls that are 70 dB SPL loud at a distance of 1 m (Bennet-Clark, 1989), and Staudacher (2009) found that the last instar nymphs of the closely related *G. bimaculatus* have a hearing threshold of 75–90 dB SPL at 5 kHz, suggesting that last instar crickets can hear calling males up to 1 m away. While it is unclear whether *A. domesticus* males produce and perceive similar amplitude calls, it seems likely that last instar nymphs could perceive male calls from 30 cm away. Therefore, our test juveniles could hear calling males during their development without physically interacting with them. We continually monitored calling in the environmental chamber over a 24-h period to note when and how much males sang. Dividing the 24 h into 30-min segments, we found that males sang throughout the dark period of the day (20 of 20 half-hour segments) and sang in 40% of the half-hour segments of the light period of the day (8 segments of 24). In addition, while it was not possible to tell from the recordings how many males were singing at any one time, more males were singing more loudly during the dark period (CM personal

observation). Using a Casella CEL-24× sound level meter, we measured the amplitude of male singing in the dark period in the range 60–70 dBA (impulse time weighting).

The lack of physical contact is important since prior physical interactions have been shown to affect aggressive behaviours (Iwasaki et al., 2006). We placed nymphs in the low-density treatment in a separate environmental chamber with no adult *A. domesticus* males. We use 'low-density' as compared to 'no density', as low-density conditions in the field would likely place conspecific males further than 1 m away, and outside of the likely hearing range of juvenile crickets. However, we have found little in the literature on field studies of *A. domesticus*, and so are basing this decision on field observations of other ground-dwelling crickets (*Gryllus* spp.—CM field observations; *Gryllodes supplicans*—Sakaluk, 1987). To investigate any potential confound of having separate incubators for the two acoustic treatment groups, we reared crickets in silence in the two incubators following these experiments and found no difference in adult calling song characteristics when measuring the same song traits in the same manner as described below ($N = 10, 16$; $p > 0.05$ for all measured characteristics; Table S5).

We monitored the individual containers daily and recorded the day when the crickets moulted into adults. Once high- and low-density crickets emerged as adults, we moved the adults from their associated chambers and placed them into a third chamber to ensure that nymphs in the low-density treatment remained isolated from male calls and nymphs in the high-density treatment did not experience an altered calling environment. Therefore, adults crickets from the two treatments were in the same calling environment where they could hear each other call. Chemical cues likely played no role as *A. domesticus* likely cannot sense airborne chemicals and require contact with a conspecific or chemical-laden substrate (Assis et al., 2016). As the calling male stimuli were less than 1 m from the developing nymphs during rearing (Bennet-Clark, 1989; Staudacher, 2009), the juveniles could likely perceive the auditory cues.

We used the same crickets for all analyses below to examine correlations between different traits within the same animal (e.g., resting metabolic rate and testes mass). The order of experiments a single male underwent was as follows—(1) respirometry, (2) song recordings, (3) aggression trials and (4) dissections. Due to reversing the cricket's circadian rhythms, we ran all tests and took all measurements during the dark cycle of the day (between 08:00 and 18:00) when crickets were most active. Before each type of trial, we measured male body mass to the nearest 0.1 mg using a Torbal Analytical Scale (AGZN120) and noted male age (defined as days since final moult).

Resting whole body respiration

To measure whole-body resting respiration rate, we used a closed-circuit respirometry system—Q-Box RP1LP Low range respiration package by Qubit. Before the start of the trials each day, we let the respirometer warm-up for 30 min and then recalibrated it using CO_2-free air, O_2-free N_2 gas and ambient outside air. Then, we further scrubbed CO_2-free air (from a 30 L G122 gas bag) of CO_2 (using a column of soda lime) and water (using two columns of Drierite). We pumped this scrubbed air (Q-P103 gas pump, Q-G266 Flow Monitor 1 LPM) at a regulated flow rate of 50 mL/min into the 59.7 mL animal chamber, with an S132 temperature probe inserted in the chamber. We again scrubbed air flowing from the chamber of water (Drierite column), and we measured the O_2 and CO_2 levels using Q-S102 O_2 Analyzer 0%–100% enhanced, and a Q-S151 CO_2 Analyzer 0–2000 ppm. We used a C901 Logger Pro software, on a Windows desktop computer, for all analyses.

Before starting each trial, we allowed the chamber to stabilize at a CO_2 level of 0 ppm. We tested 93 animals: 46 high-density males and 47 low-density males between the ages of 4- and 5-day post-emergence. Males did not call during respirometry, and locomotion was minimal. Once the test individual was placed in the chamber, we once again waited for the CO_2 level to stabilize and then closed the respirometry system, so the air in the system was recycled repeatedly through the animal chamber. At this point, the measured CO_2 level started steadily increasing, and the O_2 level started steadily decreasing. When CO_2 reached 1000 ppm in the chamber (approximately 10 min), we stopped data collection. For analyses, we used a linear section of the CO_2 (increasing with time) and O_2 (decreasing with time) graphs to calculate the volume of carbon dioxide produced (vCO_2; μL/min) and the volume of oxygen consumed (vO_2; μL/min), using the equations specific to closed-flow systems in the Logger Pro Software and the volume of air in the closed system.

Reproductive investment: mass of testes and accessory glands

To examine the effect of population density during development on the masses of testes and accessory glands, we cold euthanized and dissected 73 males (36 high-density and 37 low-density) between the ages of 6- and 16-day post-maturation. After dissecting a male, we removed and weighed the testes and accessory glands to the nearest 0.1 mg.

Male calling song characteristics

To record male calling song, we used wooden anechoic chambers (inside dimensions—30 × 55 × 55 cm) lined with 5 cm wide acoustic wedge foam (Auralex Studiofoam). We recorded males individually, using a Sennheiser ME66 shotgun microphone attached to a K6 power module, which fit through the chamber lid. We placed the male cricket, housed in its container, into the anechoic chamber for recording. We amplified male calling song from the microphones using PreSonus BlueTube V2 preamps and recorded using Cambridge Electronic Design Limited's Micro-1401-3 data acquisition unit and Spike2 (software for multi-channel continuous data acquisition and analysis) on a Windows desktop computer. Songs were digitized at 22 kHz and analysed using a custom script specific for cricket song

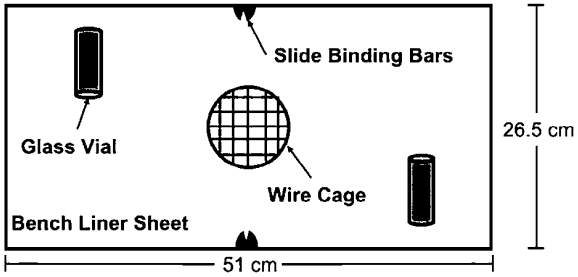

FIGURE 1 Experimental setup of the aggression trial. Graphic is the view from the top of a 32 cm tall clear glass aquarium. We slid a fitted opaque plastic sheet into the slide binding bars during the 10-min acclimation.

analysis (recording and analyses methods similar to Tolle & Wagner Jr., 2011). We visually examined the recorded period (ranging between 30 min if a male called right away, and 3 h) and selected a continuous bout of song that was at least 5 min long and analysed the chirp rate, average chirp duration, average number of pulses per chirp, average pulse duration and average dominant frequency for each male. If a male did not call, we tested them the following day. Males within the correct age range (4–11 days) were chosen randomly for recording, and recordings occurred from the start of the dark period (08:00) to the end of the dark period (18:00)—there was no significant difference in start time of recording for males in the two treatments ($F_{1,83} = 0.03$; $p = 0.866$).

We recorded males before and after aggression trials. As we found no differences in call characteristics between recordings (paired t-tests comparing before to after), we used five continuous minutes of calling song from either the first (if possible) or second recordings. We analysed the recordings of a total of 85 animals: 40 high-density males (36 recorded before aggression trials, 4 recorded after) and 45 low-density males (39 recorded before aggression trials, 6 recorded after), and all tested between ages 4- and 11-day post-emergence. Dropping the post-aggression males from the analyses yielded the same results as including them, and therefore, we only present the results for the combined dataset.

Male–Male aggressive contests

To investigate the effect of rearing density on male aggression, we staged 31 pairwise aggressive interactions using a low-density male and a high-density male for a total of 62 males tested, each tested only once. Males were between the ages of 5- and 12-day post-emergence. To conduct male–male aggression trials during the cricket's perceived night-time, we transferred the males to a room lit only by red lights—which crickets cannot perceive (Zufall et al., 1989)—to simulate night-time. We carried out trials in an aquarium (26.5 × 51 × 32 cm) bottom-lined with a white bench liner sheet to make the crickets more visible in video recording (Figure 1). We crafted two burrows from glass vials (15 mL, 21 × 70 mm) coated in duct tape to make them opaque and secured them to the bottom of the aquarium. We fashioned a round metal cage from window wire and secured it to the bottom–middle of the aquarium. The metal cage housed a mature female in 12 of the 31 trials to test the effects of female presence on male aggression. We included female presence as a covariate in models of aggressive behaviour but it had no effects (Table S1). We glued slide binding bars to the sides of the aquarium, and we slid a plastic sheet—fitted to the width of the aquarium and made to fit over the wire cage—into them to create a divider. We set a Panasonic 4 K video camera (HC-WXF991) to a night scene infrared setting in 4 K mode to record all trials.

Immediately before the trial, we marked crickets (randomly) with either a white or black mark on the pronotum with a sharpie paint pen for identification during video analysis. We placed the crickets in the aquarium on either side of the divider for a 10-min acclimation. After this, we removed the divider and recorded interactions for 15 min following the first contact of the dyad. For video analysis, we used a behavioural-logging software—Behavioural Observation Research Interactive Software (BORIS; Friard & Gamba, 2016). All 15-min trials consisted of multiple contests (when a pair were in physical contact). We defined a contest as aggressive when there was a clear winner, which occurred when an opponent withdrew from the contest. We collected the number of aggressive contests that took place, noted the winner and respective contest lengths defined from the initiation of physical contact to the loser withdrawal.

The behaviours of one opponent depend on the behaviours of the other, especially after the first couple of seconds of a contest (Alexander, 1961; Iwasaki et al., 2006). As such, we recorded the rearing density of the male that completed the first unique aggressive behaviour [from the ethogram by Adamo and Hoy (1995)] in the first aggressive contest. In addition, we recorded the rearing density of the first male to sing the aggressive song in each dyad and the time spent singing the first bout (the end defined as at least a 5-s pause) of the aggressive song for both males. We also summed the total length of time individuals sang the aggressive song over the entire 15-min trial and divided it by the sum of the contest lengths for that particular dyad (hereafter, 'aggressive song length per total contest length') to correct for the proportion of time spent in the contest.

Statistical analysis