Effects of the maternal social environment on the mating signals and mate preferences of adult offspring in *Enchenopa* treehoppers

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Abstract

Much is known about how the maternal environment can shape offspring traits via intergenerational effects. It is less clear, however, whether such effects may reach adult offspring sexual traits, with potential consequences for sexual selection and speciation. Here, we report effects of adult female aggregation density on the mating signals and mate preferences of their offspring in an insect that communicates via plant-borne vibrational signals. We experimentally manipulated the density of aggregations experienced by egg-laying mothers, reared the offspring in standard densities, and tested for corresponding differences in their signals and preferences. We detected a strong effect in male signals, with sons of mothers that experienced low aggregation density signaling more. We also detected a weak effect on female mate preferences, with daughters of mothers that experienced low aggregation density being less selective. These adjustments may help males and females find mates and secure matings in low densities, if the conditions they encounter correspond to those their mothers experienced. Our results thus extend theory regarding adjustments to the social environment to the scale of intergenerational effects, with maternal social environments influencing the expression of the sexual traits of adult offspring.

Keywords:

Behavior, evolution, interacting phenotypes theory, maternal effects, social plasticity, vibrational communication

Social environments constitute important causes of variation in animal behavior and other phenotypes, including those associated with courtship and mate choice (West-Eberhard 1983; Moore et al. 1997; Wolf et al. 1998; Valone et al. 2002; Danchin et al. 2004; Bailey et al. 2018; Rodríguez et al. 2019; Parker 1974; Bretman et al. 2011; Parker et al. 2013; Jennions & Petrie 1997; Hebets & Sullivan-Beckers 2010; Verzijden et al. 2012). These social influences are not limited to the generation in which they are experienced. In fact, across taxa there are examples of the maternal social environment having intergenerational effects on traits such as sociality and aggression (Bentz et al. 2013; Babb et al. 2014). We were interested in whether these maternal intergenerational effects could ultimately influence courtship and mate choice behavior.

Effects of the maternal social environment on offspring courtship and mate choice behavior would constitute a form of maternal effect or, more broadly, an indirect environmental effect imparted on offspring by mothers (Moore et al. 1997). We tested for such intergenerational effects to explore how the social environment can influence sexual selection across generations. Specifically, we asked whether intergenerational effects might allow adjustments to the conditions offspring will face (cf. Mousseau & Fox 1998; Marshall & Uller 2007; Bentz et al 2013; Bestion et al. 2014; Storm et al. 2010; Ensminger et al. 2018; see below). We also asked whether intergenerational effects may cause variation in male signals and/or female mate preferences, and thereby alter the signal-preference relationship and influence the strength and direction of sexual selection due to female mate choice; patterns of assortative mating; and/or the maintenance of genetic variation of sexual traits (Jennions & Petrie 1997; Bailey & Moore 2012; Rodríguez et al. 2013a; Rosenthal 2017; Desjonquères & Rodríguez 2023).

We therefore tested the hypothesis that the maternal social environment acts as a cause of variation in adult offspring mating signals and/or adult offspring female mate preferences. We

used a species that lives in aggregations as juveniles and adults, including the egg-laying stage (see below). We experimentally manipulated the egg-laying density of mothers. We then reared those mothers' offspring in standard aggregation densities, and tested for changes in male signals and female mate preferences according to egg-laying maternal aggregation density treatments. Although we did not measure any aspect of the mothers' phenotype, this experiment allows us to test for variation in adult offspring traits due to inputs into trait expression arising from the social environment of their mothers.

We based our expectations for potential adjustments in adult offspring sexual traits according to the aggregation density experienced by egg-laying mothers on theory regarding adjustments to experience of competitors and options in the recent/immediate social environment. These expectations assume that the maternal social environment is in fact informative about the conditions adult offspring will face in mate searching and mate choice (see below). Males are generally predicted to increase signaling investment when facing increased risk of sexual competition (Bailey et al. 2010; Callander et al. 2013; Parker et al. 2013; Höbel 2015), which may be conditions in which females may become more selective in their mate choice (Rebar & Rodríguez 2016). However, experimental studies have also found increased male investment (faster development, higher signaling effort) with lowered risk of competition (Bretman et al. 2011; Kasumovic et al. 2011; Rebar et al. 2016). Either form of the effect may be advantageous; e.g., making males more competitive in the former case (Parker et al. 2013) but perhaps making males more likely to find mates in low densities in the latter case (Bretman et al. 2011; Rebar et al. 2016). The prediction for male adult offspring signaling effort in our experiment must therefore remain agnostic regarding the sign of the effect, and focus on the presence of effects due to the maternal social environment.

Females may adjust mate choice decisions in different ways: they may adjust preferred mate types according to the options available, and/or they may adjust how selective they are in their mate choice (Hebets & Sullivan-Beckers 2010; Verzijden et al. 2012; Rodríguez et al. 2013c; Desjonquères & Rodríguez 2023). We do not have a rationale for anticipating change in preferred mate types according to our treatments of aggregation density experienced by egg-laying mothers. There are, however, strong reasons to expect adjustments in female selectivity. For instance, the "mating assurance" hypothesis posits that when preferred mate types are likely to be present, females can afford to be highly selective; however, when preferred mate types are likely to be rare or absent, the cost of rejecting a potential mate male is high because of the low likelihood encountering another, and females should lower their selectivity to ensure they obtain a mating (Fowler-Finn & Rodríguez, 2012a,b; Rodríguez et al. 2013c; Desjonquères & Rodríguez 2023). In terms of our experiment, the prediction is therefore that daughters of mothers that experienced low aggregation densities during egg laying will show lower selectivity in mate choice.

Methods

We investigated the transgenerational effects of maternal density on offspring traits in a member of the *Enchenopa binotata* species complex of treehoppers (Hemiptera: Membracidae). These plant-feeding insects are widely distributed across eastern North America (Wood 1993; Cocroft et al. 2008). *Enchenopa* communicate with plant-borne vibrational signals and live in aggregations as juveniles and adults (Cocroft & Rodríguez 2005; Cocroft et al. 2008). Males in search of a mate fly from plant to plant and produce bouts of advertisement signals, each

consisting of a "whine" (a pure tone which decreases slightly in frequency) followed by a series of pulses (Hunt 1994; Cocroft et al. 2008, 2010). If a receptive female finds a male's signals attractive, she responds with her own signal, which alerts him of her presence; the male and female then establish a duet that facilitates pair formation (Rodríguez et al. 2004, 2006; Rodríguez & Cocroft 2006; Cocroft et al. 2008). Thus an *E. binotata* female can decide whether to inform a particular male about her presence on the plant and allow him the opportunity to court her. This behavior of selective duetting has revealed strong mate preferences in *E. binotata* females for the features of male advertisement signals, mainly according to dominant frequency (Rodríguez et al. 2004, 2006, 2013a; Cirino et al. 2023). Selection on signals arises mainly from female mate preferences and has established a strong pattern of signal-preference coevolution across the complex (Rodríguez et al. 2004, 2006, 2013a; Cocroft et al. 2008, 2010; Sullivan-Beckers & Cocroft 2010).

Members of the *E. binotata* complex have a yearly life cycle with some variation in the timing of the mating season dictated by the phenology of their host plants (Wood et al. 1990; Cocroft et al. 2008). Mating occurs from June to mid-August, with males progressively dying off until, by late August, only females remain (Cocroft et al. 2008; Sullivan-Beckers & Cocroft 2010). Females mate only once (Wood 1993; Sullivan-Beckers & Cocroft 2010), and begin to aggregate to lay eggs in their host plants in late August. Females remain on the host plant until they die with the first frost (Cocroft et al. 2008). The treehoppers overwinter as eggs, and the flow of sap (depending on region, but typically around May) in the host plants triggers embryo development (Cocroft et al. 2008). Nymphs develop in aggregations on their plant over ca. 4 weeks and reach adulthood by late May/early June (Cocroft et al. 2008). Because embryo development and eclosion from eggs is determined by the phenology of the treehoppers' host

plants (Wood et al. 1990; Cocroft et al. 2008), life cycles are markedly synchronized for any one species at any one site; e.g., most individuals in a population eclose from eggs and reach adulthood within ca. one week of each other.

Most members of the complex have not been formally described. Nevertheless, they can be distinguished by their host plant species, nymph coloration, and adult signal frequencies (Cocroft et al. 2008; Hamilton and Cocroft 2009). We worked with the *E. binotata* species that lives on *Viburnum lentago* (Adoxaceae) host plants in Wisconsin, has nymphs with gray-green coloration, and adult male signal frequencies of ca. 165 Hz. Male signal frequency in this species is under weak directional selection, with females preferring signals of ca. 185 Hz (Rodríguez et al. 2013b, 2018; Fowler-Finn et al. 2017). We preserved all individuals used in the trials below in 95% ethanol in the Rodríguez Lab collection.

Manipulating maternal aggregation density during egg laying

We collected mated females in September of 2020 at Cedarburg Bog (Saukville, WI). By this time of the season, the majority of females have mated and no males remain in our population (pers. Obs.; cf. Sullivan-Beckers 2008). These females were ca. 10 week-old adults (with eclosion from egg around the first week of June and adult molt around the first week of July at our site; pers. Obs.).

We collected females from several *V. lentago* trees across four large copses separated from each other by ≥150 m, taking no more than 2 females from any one plant individual. As females aggregate for egg laying from a much wider dispersion than during the mating season (Cocroft et al. 2008), it is highly unlikely that any neighboring females were related.

We manipulated aggregation density for egg laying for these females in a climate-controlled room at the University of Wisconsin-Milwaukee Greenhouse (temperature: day: mean = 19.9, SD = 5.1; night: mean = 16.1, SD = 4.1; light cycle: 12:12 h). We placed females in 11 × 29 cm plastic cups each fixed around a stem of a potted exemplar of their host plant (only one cup per plant). This gave us a fixed extent of stem to manipulate aggregation density by introducing different numbers of females. We standardized plant quality by using 2-3 year-old exemplars of approximately the same size (ca. 0.7 m tall and 0.5-1cm at the base of the stem) and vigour. We assigned females at random to one of three aggregation density treatments: high, medium, or low (15, 8, and 3 females /aggregation, respectively). We based these treatments on observed variation of adult groups in the field, with the medium density treatment approximating field conditions (pers. obs). We created more replicates for the low density treatment to attempt to even final offspring sample sizes (Table 1).

We allowed females to lay their eggs *ad libitum* within the experimental aggregations throughout September and October. The range of egg masses laid by females in the experimental aggregations (low: 6-43; medium: 40-103; high: 77-126) corresponds to the range observed in the field (April 2024 survey at the UWM Field Station, adjacent the Cedarburg Bog collecting site: 1-149 egg masses/stem.

Once no female had laid eggs for 2 weeks, we brought the plants outside the greenhouse to expose them to the cool fall temperatures and trigger plant and egg dormancy. We kept eggs on the original plant, as females lay them under the epidermis of the plant and then cover them with a waxy material (Cocroft et al. 2008). Further, eggs must remain in this position for embryo development and nymph hatching to be triggered by the flow of phloem when plants exit dormancy (Cocroft et al. 2008). Females died as in the field with the first frost (Cocroft et al.

2008). When dormancy set in 4 weeks later, we brought the plants into a cold storage room at the greenhouse and kept them at 3-4 °C for overwintering.

Offspring rearing

To start embryo development, we brought the plants with eggs back into the greenhouse room in February of 2021, and gradually increased the temperature over a month to the above conditions. This brought the plants out of dormancy, and the movement of phloem brought the treehopper embryos out of diapause (Cocroft et al. 2008). Nymphs hatched in March within ca. 1 week of each other.

We transferred 2nd instar nymphs to fresh potted exemplars of their host plant that we had brought out of dormancy starting 1 month before the plants with egg masses (Figure 1). We waited until the 2nd instar stage to establish the rearing aggregations due to concerns that 1st instars might be too small and delicate to move without hurting them. This introduced a potential confound into the experiment, as the nymphs briefly experienced different aggregation densities (corresponding to their mothers' egg-laying density treatments), which can influence signals and mate preferences (Fowler-Finn et al. 2017). A related potential concern applies to embryos in the eggs. We have no experimental way to tease apart these potential effects from that of mothers' egg-laying density treatments. However, we consider that they do not represent serious problems (see Discussion).

We spread nymphs from any one maternal treatment replicate plant onto several different rearing replicate plants. All nymph aggregations had starting densities of 30 nymphs per plant, except for two replicate aggregations from the maternal low density treatment and one replicate

aggregation from the maternal medium density treatment. For these replicates, the two low density replicates were n=7 and n= 25 and the medium density replicate was n=7 (removing these replicates did not affect the significance of our results; see below). Upon the adult molt in April, we sorted the offspring by sex, transferring females onto fresh plants and leaving the males on the original plants to prevent mating and courtship experience (Figure 1).

To account for potential confounds in development stemming from our maternal treatments, we monitored nymph survivorship, the proportion of adults who became sexually receptive, sex ratio, and adult mass. We found no differences in these variables between treatments (see supplemental for details).

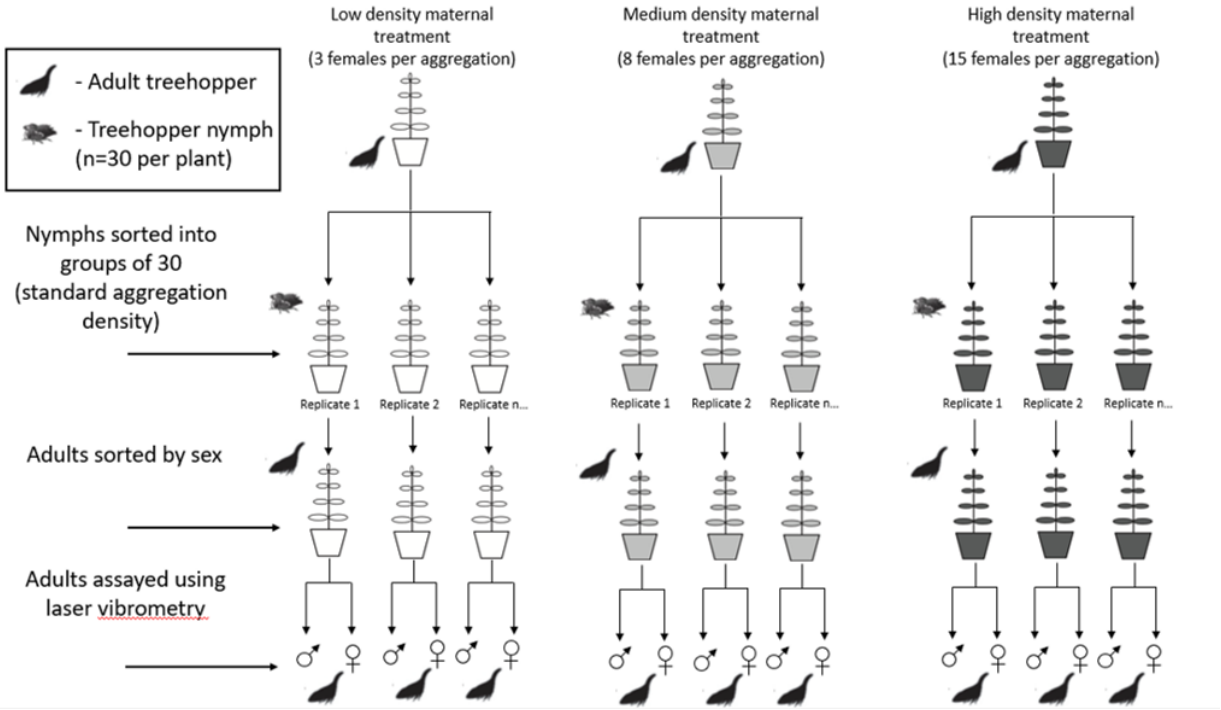

Figure 1. Outline of the experiment testing for effects of the maternal social environment with *Enchenopa* treehoppers. We randomly assigned females to replicated aggregation density treatments during egg laying. We then reared their offspring at a standard aggregation density.

Upon the adult molt, we sorted the treehoppers by sex on separate rearing plant replicates, to prevent them from experiencing courtship experience and mating.

Recording and analyzing male advertisement signals

To record male signals, we took advantage of their natural tendency to signal spontaneously when placed on a stem of their host plant (which mimics arrival on a new plant in their natural mate-searching behavior) (Cocroft et al. 2008). Drawing haphazardly from the different replicate rearing plants, we placed each male on a potted exemplar of their host plant (henceforth, the recording plant). We used a single plant for recording males (and testing females; see below) to avoid any potential confounding effects due to differences in the signal transmission features of different plant individuals (Cocroft & Rodríguez 2005). We placed all males at a standard position on the plant stem, ca. 5 cm above the recording laser dot (see below). We monitored the air temperature near the position of the male (within 40 cm) with a thermometer (catalog number 14-648-26, Fisher Scientific, Hampton, NH, USA). The mean recording temperature while recording males was 24.5C (SD = 0.66; range = 23.5-26.5C).

We recorded male signals using a laser doppler vibrometer (Polytec CLV 2534; Polytec Inc., Auburn, MA, USA) which allowed us to record vibrations transmitted along the recording plant without direct contact with, or disruption of, the substrate. We focused the beam of the laser vibrometer on a small piece of reflective tape (ca. 2 mm^2) attached to the stem of the plant. We sent the output from the vibrometer to a frequency filter (40–4000 Hz; Krohn-Hite 3202; Krohn-Hite Corporation, Brockton, MA, USA) and oscilloscope (1MB mixed signal

oscilloscope; HMO 1002; Rohde and Schwarz; Munich, Germany) and then to a MacBook Pro laptop computer (Apple; Cupertino, California) through a USB audio interface (Edirol USB Audio Capture UA-25; Roland, Hamamatsu, Japan). We recorded the signals on this computer with the program AUDACITY (v. 2.1.2; http://audacity.sourceforge.net/) at a sampling rate of 44.1 Hz. To isolate the recording set-up from building vibrations, we placed the recording plant on a pad of shock-absorbing sorbothane (Edmund Scientifics, Tonawanda, NY) on top of a 135kg iron plank resting on partially inflated inner tubes on a table. The legs of the table were on rubber pads.

We allowed each male 5 min to signal after placing him on the recording plant. If a male did not signal halfway through the allotted time, we played a primer of a male signal followed by a female response in an attempt to elicit a call (see below). If the male did not signal within the 5 min interval, we returned him to his plant and tried again on a subsequent day. We excluded males that did not signal in three such attempts. In total, we recorded 120 males (Table 1). We did not keep track of whether the proportion of males that required a primer to induce signaling varied across treatments. However, we do not expect this to introduce a confound. Prior research has found that the immediate social male-female signaling environment does not alter the features of male signals on which we focus here (mainly signals/bout and signal frequency; see below), although it may influence signal rate (Rebar & Rodríguez 2016), which did not vary across our treatments (see below).

Following recording, we assessed male signal features using the program AUDACITY. In terms of the signal-preference relationship, the most relevant signal feature is dominant frequency; this is the most distinctive signal feature across species in the *E. binotata* complex, and the one for which females have the strongest mate preferences (Rodríguez et al. 2006,

2013a; Cocroft et al. 2010). However, as possible adjustments to the level of sexual competition in the social environment most often involve signaling effort, we also assessed variation in other signal features (Figure 2).

The features of *E. binotata* male advertisement signals vary along signal bouts (e.g., increasing amplitude and length, slightly decreasing frequency) (Cocroft et al. 2010). To account for this variation, we took measurements from a standard "landmark" position along the recorded signal bouts: the third signal of the first bout. If a bout contained less than three signals, we measured the last signal.

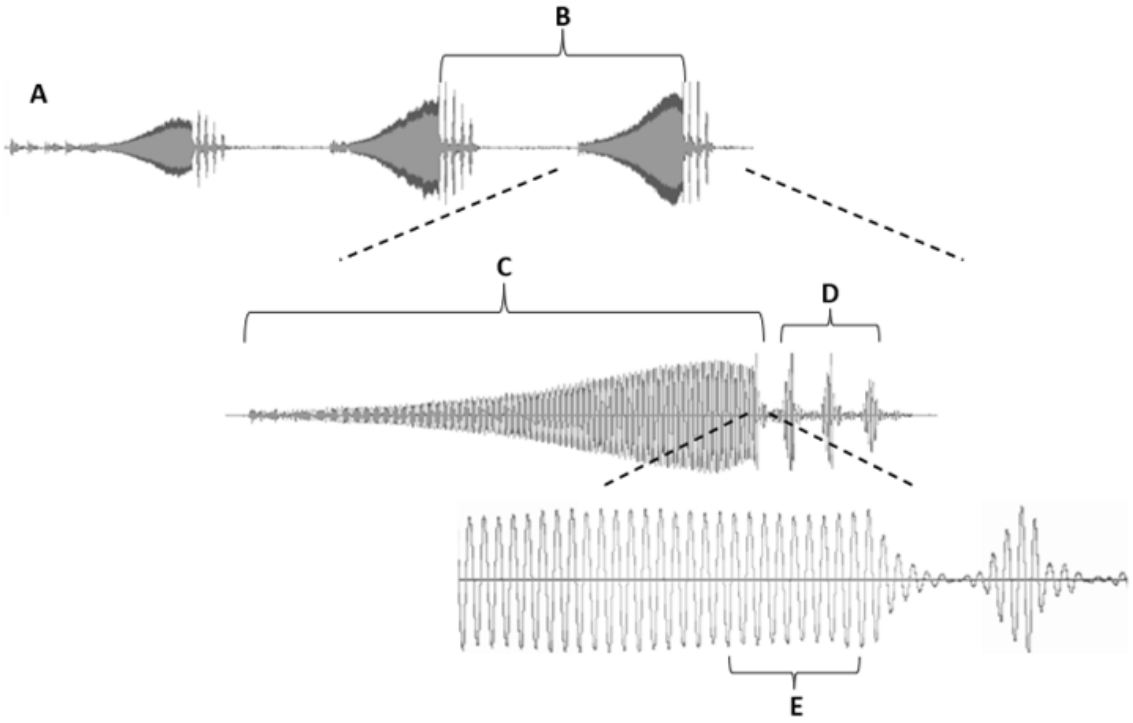

Figure 2. Male *E. binotata* advertisement signal features examined in this study. **A**: Signals per bout. **B**: Inter-signal interval. **C**: Whine length. **D**: Pulse number and pulse rate (pulse

278 number/interval between first and last pulse). **E**: Dominant frequency (estimated from the length
279 of 9 wave cycles at the end of the whine, which is the section of highest amplitude).
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281
282 *Vibrational playbacks and describing female mate preferences*
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284 Describing variation in mate preferences requires several trials with each individual (see below),
285 and with too many trials females may become unresponsive. We therefore focused on mate
286 preferences for only one signal feature: the dominant frequency of male signals. These are the
287 strongest mate preferences in the *E. binotata* complex, and signal frequency is the most
288 distinctive adult phenotype among species in the complex (Rodríguez et al. 2006, 2013a; Cocroft
289 et al. 2008, 2010).
290 To assess mate preferences, we took advantage of the natural behavior of *Enchenopa*
291 females of selectively duetting with males they find attractive (Rodríguez & Cocroft 2006;
292 Cocroft et al. 2008). A mate-searching male that does not receive a duetting response from a
293 female will not be alerted about her presence and will fly off to another plant to signal. By
294 contrast, a male that is engaged in duetting by a female will remain on the plant, search for the
295 female, and continue duetting until mating begins—*Enchenopa* females control which males
296 they encourage to court them and which remain ignorant of their presence. *Enchenopa* female
297 selective duetting thus provides a realistic and convenient assay of their response to signals
298 (Rodríguez et al. 2004, 2006, 2013a; Cocroft et al. 2008).
299 We presented females with synthetic vibrational playback stimuli varying in frequency.

We generated the stimuli and controlled their presentation from an iMac desktop computer (A1208; Apple; Cupertino, CA) with custom MATLAB (version 7.5.0.338; MathWorks, Natick, MA; http://www.mathworks.com) scripts. We calibrated the amplitude of the playback stimuli to 0.15 mm/s using the oscilloscope. We imparted the stimuli onto the recording plant with a DC unit connected to a piezo controller (MDT694A; Thorlabs, Newton, NJ) that drove a piezoelectric stack attached to the stem of the plant with accelerometer wax (Model 32227 mounting wax, Endevco, San Juan Capistrano, CA, USA). We recorded the playback stimuli and female response signals with the laser vibrometer as described above.

To begin a playback trial with a female, we placed her on the recording plant at a standard position on the stem, ca. 5 cm above the laser dot, and allowed her 2 min to settle. We drew haphazardly from the different replicate rearing plants. We tested whether the female was sexually receptive with a primer playback (a recording of a male signal approximating mean population features). If the female did not duet with this, we put her back on her rearing plant and tested her another day. We excluded females that did not duet in three such attempts.

We presented sexually receptive females with 18 playback stimuli in random sequence, ranging in signal frequency from 140-250Hz. This frequency range slightly exceeds the range of signal frequencies in the species, in order to capture the full shape of the preference (Kilmer et al. 2017). All the other features of the stimuli were set to the population mean: each stimulus had 3 signals/bout; inter-signal duration of 3170 ms; whine length of 700 ms; four pulses per signal; and pulse rate of 17.4 pulses/sec (Desjonquères et al. 2023).

We recorded the vibrational playbacks and female duetting responses using the above laser vibrometry set up, with the program AUDACITY on the MacBook Pro. We monitored the air temperature near the position of the female (within 40 cm) with the thermometer. The mean

323 recording temperature while recording females was 24.6C (SD = 0.50; range = 23.5-25.5C). We
324 completed playback trials for 192 females (Table 1).

325 Our assay of female response was the number of duetting responses that females
326 produced to each of the playback stimulus bouts (ranging from 0-3; i.e., from responding to none
327 to responding to all 3 signals in the playback bouts; see above). We noted female responses to
328 the playbacks from the waveform of the playback recordings with the program AUDACITY.
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331 *Statistical analysis*

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333 Females in the *E. binotata* complex mate only once (Wood 1993; Sullivan-Beckers & Cocroft
334 2010). Consequently, each female's offspring in the egg-laying and nymph-rearing host plant
335 replicates constitute a full-sibling family, mixed in with the offspring of the other females in the
336 replicate. This introduced an element of non-independence in the data that was impossible to
337 account for, as we had no way to track egg and offspring families along the experiment. One
338 concern is that, as treatments consisted of different numbers of egg-laying females (Table 1),
339 they may have varied in the degree of mixing of related individuals and corresponding data
340 dispersion. For example, there may be genetic variation in survivorship, or in how survivorship
341 varies with aggregation density.

342 We attempted to deal with such potential problems in two ways. First, we reared all
343 nymphs at the same aggregation densities to attempt to mitigate any affect caused by juvenile
344 density. We also attempted to equalize final sample sizes by creating more replicates for the low
345 maternal aggregation density treatments (see supplemental). Thus, the mixtures of related and

unrelated individuals in each replicate and treatment were likely similar, and unlikely to bias the results. Additionally, we accounted for plant replicate identity in the analyses with random terms (see below), which partly covers non-independent sibling data points. We therefore consider that the problem of non-independence was likely weak and diluted similarly across the treatments in our experiment, and that it only suffered from an unavoidable but low level of pseudoreplication that was unlikely to strongly force spurious significance in the analyses.

Testing for effects of the maternal social environment on male adult offspring advertisement signals

To analyze variation in male signals, we first examined whether the different signal traits we measured (Figure 2) were strongly related to each other with Pearson correlations. The purpose of this preliminary analysis was to assess the risk of spurious significance from testing with many highly correlated traits (cf. Rice 1989). We found that most correlations were weak and non-significant ($r \leq 0.22$, $P \geq 0.05$) and one was significant and of moderate effect size (the pulse rate-pulse number correlation: $r = -0.42$, $P < 0.001$). This suggests that the risk of spurious significance from including all signal traits in the analyses detailed below is low.

We therefore tested for maternal effects on signals with separate linear mixed models for each signal trait. Each model had the following explanatory terms: treatment and recording temperature as fixed effects; and plant replicate nested within treatment as a random term. As each male contributed a single data point for each signal trait, the models did not have a random term for individual identity. We ran these analyses in R using the package glmmTMB (version 1.1.8) and correlation (version 0.8.4).

Testing for effects of the maternal social environment on female adult offspring mate preferences

We analyzed variation in female mate preference functions with a function-valued approach (Ritchie 1996; Meyer & Kirkpatrick 2005; Fowler-Finn & Rodríguez 2012a,b; Stinchcombe et al. 2012; Kilmer et al. 2017). We used a generalized linear mixed model with the number of responses (0-3) of each female to each stimulus as the dependent term (modeled as an ordinal variable with a Poisson error distribution using the glmmTMB package in R). We included the following fixed explanatory terms in the model: treatment; linear and quadratic components for stimulus frequency; the interactions between treatment and these linear and quadratic stimulus frequency terms; and recording temperature. We also included random terms for rearing plant replicate (nested within treatment); and female identity (nested within treatment and replicate, as each female contributed multiple data points across her response curve to the stimuli).

In this model, the main term for treatment tests was for overall differences in intercept (or elevation) between mate preferences across treatments. The main terms for stimulus frequency (linear and quadratic) test for overall linear slope and curvilinear shape components in the mate preferences. The interactions between treatment and the stimulus frequency terms (linear and curvilinear) test for differences in the shape of the mate preferences across treatments (i.e., differences in slope with the linear term, differences in curvature with the quadratic term).

We assessed the effect size of significant terms (Cohen 1988; Nakagawa & Cuthill 2007) with the measure partial eta squared (η^2_p), which we estimated following Lakens (2013). We also converted η^2_p to a measure of effect size (r) that is bounded between 0-1 and has intuitive standard categories of small ($r < 0.30$), medium ($0.30 < r < 0.50$), or large ($r > 0.50$) magnitude (Cohen 1988; Nakagawa & Cuthill 2007), thus: $r = \sqrt{\eta^2_p}$ (cf. Lakens 2013).

Results

Effects of the maternal social environment on male offspring advertisement signals

The number of signals/bout varied significantly across treatments (Table 1), with males whose mothers experienced lower density aggregations during egg laying producing more signals/bout (Figure 5). This represents a large difference across treatments (effect size of main treatment term: $\eta^2_p = 0.287$; $r = 0.54$). None of the other signal traits varied significantly across treatments (Table 1; Figure 3).

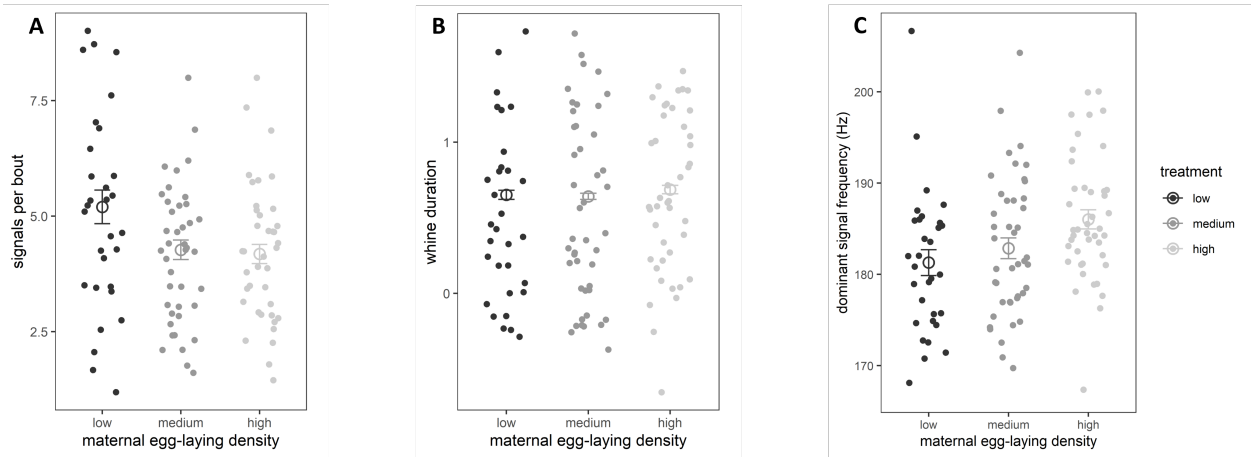

Figure 3. Variation in *Enchenopa* male signal traits across treatments in the experiment testing for effects of the maternal social environment. Here we show results for the signal traits that we discuss in terms of signaling effort and the signal-preference relationship: signals/bout (**A**); whine length (**B**); and dominant frequency (**C**). Open symbols with error bars indicate means ± 1 SE. (Note that the top 6 values for signals/bout in the low density treatment correspond to 4 different replicates.)

411 *Effects of the maternal social environment on female offspring mate preferences*

412

413 We found differences in the shape of female mate preferences across treatments (Table 2:
414 significant treatment × quadratic stimulus frequency interaction), with daughters of mothers that
415 experienced low density egg-laying aggregations having lower selectivity; i.e., their preferences
416 were broader and flatter in shape and had a lower overall elevation (Figure 4). This difference
417 across treatments was of small effect size ($\eta^2_p = 0.0041$ and $r = 0.06$ for the treatment × quadratic
418 stimulus frequency interaction term, which tests for differences in the preference function
419 curvature). Females whose mothers experienced low density aggregations during egg laying also
420 appeared to have a shift in peak preference towards a lower frequency (Figure 6). However,
421 when we tested only those females we found no significant slope or curvature (linear and
422 quadratic stimulus frequency terms: $F \leq 1.31$, $P \geq 0.25$; same generalized linear mixed model as
423 in Table 2 but excluding the high and medium density treatments). Thus, females whose mothers
424 experienced low density aggregations during egg laying are best viewed as having "flatter"
425 preference functions rather than lower peak preferences.

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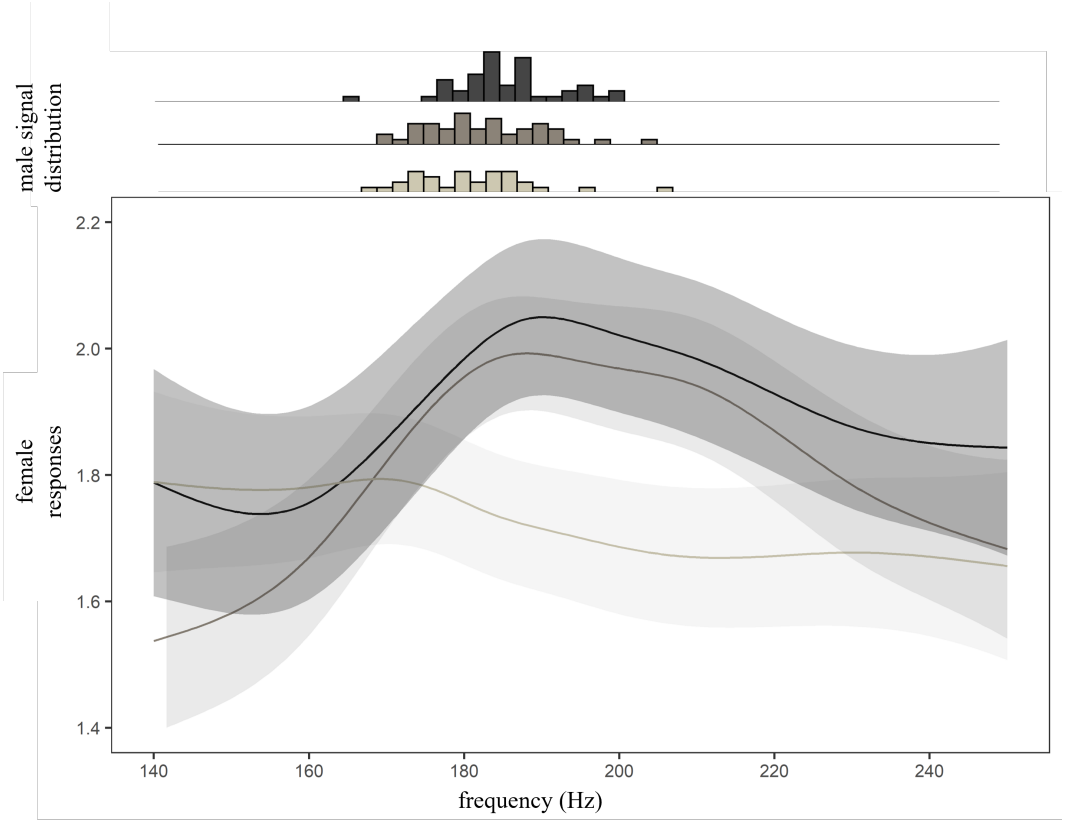

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428 *Figure* 4. Treatment-level *Enchenopa* female mate preference functions and male signal
429 frequency (histograms) in the experiment testing for effects of the maternal social environment.
430 Black: high-density; dark grey: medium-density; light grey: low-density. Ribbons: standard error
431 of the functions.

432

433 **Discussion**

434

435 We manipulated the aggregation density of egg-laying females to determine whether the
436 maternal social environment could influence the sexual traits of their offspring. We found that
437 adult offspring male signals and female mate preferences varied according to the maternal egg-
438 laying density treatments. (Our manipulation of the aggregation density of egg-laying females

439 extended to the social environment experienced by embryos and briefly by very young nymphs,
440 introducing a potential confound. We discuss below why we consider it does not present a
441 serious problem.)

442 We based our expectations for the form of these changes on theory regarding experience
443 of competitors and mating partner options in the recent/immediate social environment. These
444 mainly concern male investment in competition (e.g., signaling effort) and female selectivity. We
445 had no expectation regarding the form of the effect of the maternal social environment on adult
446 male offspring signaling effort, as increased effort under conditions indicating likely high or low
447 competition may be advantageous (Bretman et al. 2011; Parker et al. 2013; Rebar et al. 2016).
448 We found that sons of mothers that experienced conditions that might indicate low availability of
449 mates (low egg-laying aggregation density) showed increased signaling effort (signals/bout,
450 albeit not signal length). This may make males more likely to succeed in finding and attracting
451 scarce mates under conditions of low density (cf. Rebar et al. 2016). In the *E. binotata* complex,
452 female mate preferences for signals/bout are weaker than for signal frequency, but females do
453 favor higher signal numbers (Rodríguez et al. 2006; although we have not characterized this
454 preference for our study species). Further, males that produce more signals/bout may also engage
455 in higher overall mate searching efforts and thus benefit in low population densities.

456 We had a stronger rationale regarding the form of the effect of the maternal social
457 environment on adult female offspring mate preference selectivity. We expected that maternal
458 egg-laying conditions that might indicate low likelihood of the presence of preferred mate types
459 (i.e., low egg-laying aggregation densities) should result in decreased selectivity. This was the
460 form of our finding: daughters of mothers that experienced low egg-laying aggregation densities
461 showed decreased mate preference selectivity. This may help females balance the search for

preferred mate types with the need to secure a mating (cf. Fowler-Finn & Rodríguez, 2012a,b; Rodríguez et al. 2013c; Desjonquères & Rodríguez 2023). Lower selectivity preferences were also less "responsive" overall (lower mean curve elevation; Figure 4). However, those females were nevertheless producing nearly 2 duetting signals in response to each stimulus (Figure 4). Thus, they would still secure approach by males, just being less selective about which males receive more responses.

The above interpretations assume that the maternal social environment is at least somewhat predictive of the conditions adult offspring will face in mate searching and mate choice, as is often the case for young offspring across animal groups (Mousseau & Fox 1998; Marshall & Uller 2007; Bentz et al 2013; Bestion et al. 2014; Storm et al. 2010; Ensminger et al. 2018). For *Enchenopa*, there is evidence that is at least partly the case. *Enchenopa* treehoppers do not disperse very much during development, and reach the adult molt on the plant (and likely stem) where their mothers laid eggs (Cocroft et al. 2008). Further, as mate-searching adults they do fly from plant to plant, but not across large distances, with flights occurring often from one part of the plant to another (Cocroft et al. 2008). It will be interesting to ask whether the maternal social environment is broadly predictive of the conditions of adult offspring, and whether this shapes the evolution of maternal and intergenerational effects (Moore et al. 1997).

We were also interested in whether the intergenerational effects we detected might influence selection on male signals due to mate choice. We detected no change in male dominant signal frequency and no change in female peak preferences for this trait, which are the most divergent phenotype among adults in the *E. binotata* complex, and the signal trait for which females have the strongest mate preferences (Rodríguez et al. 2006, 2013a; Cocroft et al. 2008, 2010). Thus, the effects of the maternal social environment that we detected did not alter the

form of the signal-preference relationship and seem unlikely to alter the form of selection on signals stemming from mate choice. However, they seem likely to affect the strength of assortative mating and the maintenance of variation in signals. With males becoming potentially more effective at mate location and attraction (regardless of the main trait under choice, signal frequency), and females becoming less selective under conditions of low density, the effects we detect may weaken both the strength of selection on signals due to mate choice and assortative mating. This may in turn help maintain phenotypic and genetic variation in male signals (cf. Chaine and Lyon 2008; Morris et al. 2010; Fowler-Finn & Rodríguez 2012a,b; Rodríguez et al. 2013c; Desjonquères & Rodríguez 2023). This does not rule out the possibility that in other animals maternal/intergenerational effects may alter the form of sexual selection (Jennions & Petrie 1997; Bailey & Moore 2012; Rodríguez et al. 2013a; Rosenthal 2017; Desjonquères & Rodríguez 2023). We hope our results will provide motivation for such exploration.

All together, we interpret our results as providing tentative support for effects of the maternal social environment on adult offspring mating signals and female mate preferences. Several aspects of our study may have made it difficult to detect the effects we were interested in, however. First, as we assembled the rearing aggregations with 2nd instars (see above), the offspring in our experiment briefly experienced differences in aggregation density corresponding to their mothers' egg-laying density treatments. Thus, our manipulation of the maternal social environment is confounded by the offspring's own very early social environment. We consider, however, that this confound was weak if at all present. Prior work has found that variation in social aggregation density along juvenile development does not influence adult male signals and influenced adult female peak preference but not preference selectivity (Fowler-Finn et al. 2017)—i.e., the reverse of what we find in the present study, thus the potential confound is

unlikely to have forced our results. Second, a related potential concern is that the embryos in the eggs may have experienced cues of the aggregation density of their mothers. We also consider this unlikely, as females do not produce any substrate-borne signals during the egg-laying season (indeed, not since after mating) (Cocroft et a. 2008; D. W. Little unpublished). Egg-laying females do produce aggregation pheromones (Cocroft et al. 2008) but those are unlikely to reach through the waxy covering and plant epidermis to reach the eggs. Third, the effects of the maternal social environment on adult offspring traits may be expected to be subtle. Studies in other species suggest that the strength of such effects may dwindle over the lifetime of offspring, and perhaps not even be noticeable in adult offspring (Lindholm et al. 2006). Thus, although our sample sizes provided statistical power to detect some effects, power may nevertheless have been limited. Fourth, effects from the maternal social environment likely interact with additive and non-additive components of direct and indirect genetic variation in mothers and offspring (i.e., as formalized in interacting phenotypes theory: Moore et al. 1997; Wolf et al. 1998; Radwan 2008; Bailey & Moore 2012; Bailey et al. 2018; Rodríguez et al. 2019). These effects may further interact with non-genetic paternal effects (e.g., Crean & Bonduriansky 2014; Crean et al. 2014; Simmons & Lovegrove 2019), which may themselves involve social components (Crean & Bonduriansky 2014). However, we expect that our manipulation of the maternal post-mating social environment is unlikely to have coincided with such potential effects. Finally, due to our experimental design, we were unable to address our hypothesis while also tracking egg and offspring families. As a result, there was an element of data non-independence. Accounting for such possible effects and interactions may facilitate detecting the (likely subtle) effects of maternal environments on adult offspring, albeit at the cost of requiring more complex experimental designs and larger sample sizes.

Our results raise questions about the mechanism(s) that may be responsible for the effects we observed. Our manipulation of mothers' social density may have influenced their endocrine system, ultimately influencing their offspring's behavior. Such an effect may involve pheromones that mothers may deposit in their eggs. For instance, it may have involved hormones circulating in the mothers' bodies at the time of the production or laying or their eggs, as in *Pogonomyrmex rugosus* ants, where caste determination involves an interplay of different hormones within the body of the queen prior to laying (Libbrecht et al 2013). Alternatively, it may have involved hormones deposited in the waxy secretion with which females cover their egg masses (Cocroft et al. 2008). If this is true, these pheromones may also act as a cue for females. Exploring the mechanisms involved in the effects we observe should help understand their regulation and adaptive value (if any), as well as perhaps point the way to more powerful manipulations.

In short, we find that the maternal social environment can have far-reaching intergenerational effects, existing even to male mating signals and female mate preferences in adult offspring. Exploring such effects across animals may also help understand variation in the form and strength of natural and sexual selection and in patterns of reproductive isolation; as well as potential adaptive evolution of plasticity arising from multiple aspects of the social environment.

Table 1. Analysis of variation in *Enchenopa* male offspring advertisement signals in the experiment testing for effects of the maternal social environment. We report Wald Chi-square tests for the fixed effects (see full description of the linear mixed models in main text). Significant terms highlighted in bold. (Recording temperature did not differ between treatments:, $P = 0.74$) (Random terms not shown.)

Signal trait	***Term***	***df***	χ^2	***P***
Frequency	Treatment	2	0.95	0.62
	Temp	1	27.99	**<0.0001**
Inter-Signal Interval	Treatment	2	0.16	0.91
	Temp	1	0.01	0.89
Pulse Rate	Treatment	2	1.85	0.39
	Temp	1	2.09	0.14
Pulse Number	Treatment	2	1.34	0.50
	Temp	1	1.32	0.25
Whine Length	Treatment	2	1.78	0.40
	Temp	1	7.08	**0.007**
Signals/Bout	Treatment	2	**7.75**	**0.02***
	Temp	1	1.76	0.18

*removing the low sample size replicates (see above) did not later the significance of this term (P= 0.02)

Table 2. Analysis of variation in treatment-level *Enchenopa* female preference functions in the experiment testing for effects of the maternal social environment. We report the Wald Chi-square test p-values for the fixed terms (Random terms not shown.)*

Term	***df***	χ^2	***P***
Treatment	2	9.37	0.91
Linear stimulus frequency (freq)	1	3.00	0.08
Quadratic stimulus frequency ($freq^2$)	1	2.96	0.08
Treatment × freq	2	**9.39**	**0.009**
Treatment × $freq^2$	2	**8.87**	**0.01 ****

* We also ran a model as in this table but also including interactions between temperature and the linear and quadratic stimulus frequency terms, to account for a potential effect of temperature on the shape of the preferences: temperature × freq: *P*-= 0.11; temperature × $freq^2$: $P = 0.14$. The result for the treatment × $freq^2$ term ($P = 0.01$) and treatment × freq term ($P = 0.007$) did not change with inclusion of those terms. It did, however, affect the main treatment term which became marignally significant ($P = 0.09$).

**removing the low sample size replicates (see above) did not later the significance of this term (P= 0.01)

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Data availability

Data has been submitted and accepted to Dryad. (https://doi.org/10.5061/dryad.bcc2fqzk8)

Author contributions

D.L. and R.L.R. conceived and designed the study. D.L. conducted the experiment and collected the bioacoustic data. D.L. and K.L. extracted the data from the recordings. D.L. and R.L.R. conducted the statistical analyses. D.L. and R.L.R. wrote the manuscript. All authors contributed to revising the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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AI statement

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