Received: 3 September 2024 | Accepted: 30 April 2025

DOI: 10.1111/een.70002

ORIGINAL ARTICLE

Ecological Entomology

Royal Entomological Society

Salty suitors: High larval sodium intake reduces adult lifespan and influences reproductive behaviour in a lepidopteran herbivore

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Funding information
Texas Ecolab, Plateau Land and Wildlife Management; National Science Foundation

Associate Editor: Carolina Quintero

Abstract

1. One potential predictor of adult reproductive success is physical condition, which can be influenced by earlier developmental stages, such as nutritional limitations during juvenile growth. Sodium is a vital element for animals, essential for physiological function and individual-level development and behaviour. When animals are sodium-limited, they at times engage in behaviours such as cannibalism or geophagy to obtain sodium. At high levels, sodium may be toxic and result in adverse physiological effects. How dietary variation in sodium availability during larval stages influences adult mating behaviour and reproductive output has received relatively little attention. Previous studies within Lepidoptera have reported varied results.
2. We used the bordered patch butterfly, *Chlosyne lacinia* (Geyer, 1837; Nymphalidae), to investigate the role of larval dietary sodium uptake on adult body size, lifespan, mating behaviour and reproductive success.
3. Body size did not differ between treatments. Individuals raised on host plants with high-sodium concentrations had shorter lifespans than those reared on low-sodium host plants, indicating that sodium in high concentrations in larval diets can be harmful. Females raised on lower sodium diets took longer to start mating regardless of males' larval access to sodium, but copulation durations did not differ. Males and females raised on host plants with higher sodium concentrations mostly did not pair differently than individuals raised on host plants with lower sodium concentrations and did not differ in their immediate reproductive success. Females raised on high-sodium host plants avoided mating with males raised on high-sodium host treatments, suggesting that larval sodium acquisition could influence reproductive decisions made by adults.
4. Reproductive success in insects is shaped by both intrinsic behavioural strategies and extrinsic environmental factors. During mating, individuals make complex choices to maximise fitness, and environmental conditions experienced during

juvenile stages—such as dietary sodium exposure—can influence reproductive decisions in adulthood. Understanding the role of sodium in these life-history traits is increasingly important in light of global ecological change: drought-driven reliance on saline irrigation is accelerating soil salinisation worldwide, which may in turn disrupt insect–plant interactions and destabilise multi-trophic dynamics. Collectively, these findings underscore how adaptive behaviours and environmental stressors interact to shape insect populations in a rapidly changing world.

KEYWORDS
behaviour; larval diet, Lepidoptera, life-history traits, mate choice, sexual selection

INTRODUCTION

Physical condition is a major explanatory factor in determining reproductive success. In some species, males or females may prefer larger members of the opposite sex, or they may prefer courtship displays that only individuals in good condition can perform (Herdman et al., 2004; Chargé et al., 2010). One reason for variation in physical condition is an individual's diet throughout its life. For example, food limitation during juvenile development can have lasting effects into adulthood. In the European earwig, *Forficula auricularia* (Linnaeus, 1758; Dermaptera: Forficulidae), food restrictions during larval development resulted in smaller adult body size and reduced clutch size in females (Wong & Kölliker, 2014). Beyond food limitation, individuals need to acquire the correct balance of nutrients, some of which may be limited in the environment. One often limited element is sodium.

Sodium is a vital element in animals because sodium pumps regulate membrane potentials and Na/K ion concentration gradients from extracellular fluids to inside cells, mediating water balance (Doughty et al., 2016). Accordingly, sodium can play an important role in reproduction and survival (Pivnick & McNeill, 1987). However, sodium is often a limited element in terrestrial ecosystems, although levels of sodium are on the rise due to drought (Liu et al., 2020). In any case, its availability varies, leading to variation in sodium concentrations among plants (Santiago-Rosario et al., 2022). Herbivores often maintain substantially higher sodium levels in their tissues than their host plants, which is the physiological underpinning of sodium limitation in herbivores. Conversely, omnivores and carnivores consume animal tissue that is already concentrated in sodium and are generally not sodium limited (Kaspari, 2020). To obtain enough sodium, some herbivores engage in behaviours such as geophagy or puddling (Bravo et al., 2008; Molleman, 2010). Puddling occurs when insects, often lepidopterans, obtain otherwise limited elements such as sodium from mud, faecal matter, urine or other excretions (Molleman, 2010). For some lepidopteran species, puddling by adults plays a role in mating because males transfer some of the acquired nutrients (such as sodium) to females during copulation, either through a spermatophore or seminal fluids (Molleman, 2010). In herbivorous lepidopterans, larval host plants also provide a source of sodium (Mitchell et al., 2020). Although the physiological function of sodium in animals is well known, the influence of sodium availability during the juvenile stage on adult mating behaviour and both short-term and lifetime reproductive success is less clear (Molleman, 2010).

Individuals' larval and adult diet, as well as sex, can influence adult lifespan, mating behaviour and mating performance, but the empirical results are mixed. Concerning dietary sodium specifically, most lepidopteran studies have focused on the acquisition of sodium at the adult stage. In other insect species, however, research has shown that diet during larval development has lasting and irreversible effects on adult performance (Barrett et al., 2009). Additionally, sodium accumulation can vary across species and between sexes within species. Santiago-Rosario et al. (2024) found that males of three lepidopteran species accumulated more sodium than females of the same species, but this pattern did not hold for the monarch butterfly (*Danaus plexippus* [Linnaeus, 1758]; Lepidoptera: Nymphalidae). In the butterfly *Chlosyne lacinia*, larvae whose diets are deficient in sodium tend to compensate through larval cannibalism (Santiago-Rosario et al., 2023), but the effect of variation in sodium on adults' behaviour was not determined. In *Thymelicus lineola* (Ochsenheimer, 1808; Lepidoptera: Hesperiidae), Pivnick and McNeill (1987) found that adult access to sodium (ranging from 0.001 to 0.5 M Na solutions) significantly increased a male's total number of matings, and the females that mated with males that had access to sodium as adults laid more viable eggs than females that mated with males without access to additional sodium. By contrast, Molleman et al. (2004) did not find a difference in the number of eggs produced between *Bicyclus anynana* (Butler, 1879; Lepidoptera: Nymphalidae) females mated to males with sodium added to their adult diets (9 g Na/1 L of water) and those with no sodium added. Both species, *T. lineola* and *B. anynana*, puddle and are polygynous, and both males and females mate multiple times, and the male provides the female with a spermatophore during copulation (Molleman et al., 2004; Pivnick & McNeill, 1987). *Thymelicus lineola* males transfer as much as 32% of their body sodium to the female during copulation, and females in turn transfer up to 50% of their body sodium to their eggs (Pivnick & McNeill, 1987). Males, then, can attempt to decrease the probability of the female remating (increasing his chance of paternity) with a higher quality spermatophore. Choosiness could be favoured in either males or females because of the high investment into copulation (for the male) and offspring (for the female). These studies show that the effect of sodium on lepidopterans may vary among species. However, the studies that examined how sodium affected reproductive success focused on sodium acquisition during the adult stage. This leaves open the question of how sodium acquisition during larval development in these species characterised by ontogenetic niche shifts (e.g., only larvae feed on leaf tissue) may influence adult behaviour and reproductive success. Additionally, there is the possibility that sodium becomes detrimental to individuals at higher concentrations, an effect called hormesis (Calabrese & Baldwin, 2003). Hormesis occurs when a chemical has a beneficial response at low doses, but beyond the optimum dose causes an inhibitory or even toxic effect as doses increase (Calabrese & Baldwin, 2003). Hormesis could occur in Lepidoptera exposed to the full range of sodium encountered in nature. However, to our knowledge, the effects of larval access to sodium on adult lifespan, mating behaviour and reproductive success have not been determined for any lepidopteran.

Our objectives were to study the influence of variation in sodium in larval diet on the adult lifespan, mating behaviour and reproduction of *C. lacinia* by manipulating the sodium concentrations in their host plants (*Helianthus annuus* L.; Asteraceae). We used two larval diet treatments: low sodium and high sodium. We predicted (1) that individuals that were raised on high levels of sodium as larvae would have a reduced adult lifespan compared to individuals that were raised on low-sodium host plants, possibly due to physiological stress at high sodium concentrations, based on previous studies (Santiago-Rosario et al., 2023). We predicted (2) that females with access to low sodium as larvae would prefer males that had access to higher levels of sodium as larvae. We also predicted (3) that females exposed to high sodium as larvae would be less choosy because they had sufficient access to sodium in their own larval diets. We also expected (4) that females exposed to high sodium would have higher reproductive success than females exposed to low sodium, but that females exposed to low sodium as larvae might compensate if they have access to sodium from males raised on high-sodium diets (i.e., an interaction effect between female larval diet and male larval diet on adult females' reproductive success).

METHODS

Study organism

C. lacinia's geographic range extends from northern Argentina into the southern United States (Kons Jr., 2000; Phelps et al., 2023). The larvae feed gregariously and are oligophagous on host plants in the tribe Heliantheae (Asteraceae) until the 4th or 5th instar, whereupon groups disaggregate, and individuals generally pupate away from others (Drummond III et al., 1970; Santiago-Rosario, 2021). *C. lacinia* is sexually monomorphic in colour and wing patterning, but females are generally larger in size, although there is significant size overlap and individuals cannot reliably be sexed using size alone (Phelps, 2024). Males usually eclose earlier than females and are ready to mate upon eclosion. Females eclose 24–36 h later than males and are sexually mature after another 24–48 h (Drummond III et al., 1970). Females tend to oviposit 25–450 eggs in clusters (Phelps et al., 2023), and eggs take ~10 days to hatch (MCV, pers. obs.).

In nature, *C. lacinia* males patrol hilltops to encounter receptive females because females congregate on hilltops to seek out mating opportunities (Alcock & Dodson, 2008). Males may hold perches (e.g., prominent branches of vegetation) or actively patrol the area looking for females (Alcock & Dodson, 2008). Currently, it is not known whether females prefer specific traits in males or whether some males are more successful in mating. Detailed observations of their mating behaviour in the field have not been published, but in laboratory conditions we have observed that males 'flutter' around females, whereby a male flies close to a female and beats his wings very quickly (Phelps et al., 2023, MCV, pers. obs.). A male will flank the female while facing the same direction and attempt to move the end of his abdomen to meet the end of the female's abdomen, twisting his abdomen sharply, at almost a 90-degree angle (video in Figure S1). The female can accept a mating by bringing her wings together over her back and allowing the male to move close enough for genital contact. If genital contact is made, the male then turns to face the opposite direction. Alternatively, she may keep her wings open and beat them periodically to rebuff the male or fly away from the male (video in Figure S1; MCV, pers. obs.).

Collection locations and colony maintenance

We collected *C. lacinia* caterpillars on two separate occasions, referred to as 'Colony 1' and 'Colony 2' for two different behavioural experiments (see below regarding Choice and No Choice trials). A diagram of the experimental design is available in Figure 1. Colony 1 was used for the Choice trials, and Colony 2 was used for the No Choice trials. We collected *C. lacinia* caterpillars for Colony 1 from eight different locations in southern Texas in April 2023 (Figure 2a; Table S1). Colony 2 caterpillars were collected from 12 locations in southern Texas in June 2023 (Figure 2a, Table S1). The following methods were followed for both Colony 1 and Colony 2. Each location was labelled as a distinct population, and we maintained each population separately. We collected 3rd–5th instar larvae (Figure 2b) to minimise the development time individuals would spend in the laboratory. We kept each population in a peanut butter jar (16.5 cm height, 8.5 cm diameter). We maintained caterpillars in their respective jars, feeding them ad libitum *H. annuus* L. (Asteraceae) or *Ambrosia trifida* (L., 1753; Asteraceae) leaves sourced locally in Louisiana. We checked the jars daily, adding more food and removing frass as needed. We separated any pupating individual into its own smaller individual container (6 cm height, 7 cm diameter), with filter paper in the bottom and subsequently checked daily for eclosion. Upon eclosing, individuals were sexed. Although there is some size dimorphism, there is substantial overlap, and accurate sexing requires viewing the external genitalia. Females have a genital pore, or ostium, visible near the ventral tip of the abdomen (Figure 2c). Males have smaller abdomens, and each has visible valves (Figure 2d). Once sexed, we uniquely numbered individuals on the left hind wing with a fine-tipped permanent marker (Figure 2e). We segregated these F0 males and females into same-sex butterfly cages (40 × 40 × 60 cm) to prevent unplanned mating. We brought F0 males and females together to mate from different populations to avoid inbred lines. Populations from Colony 1 were never

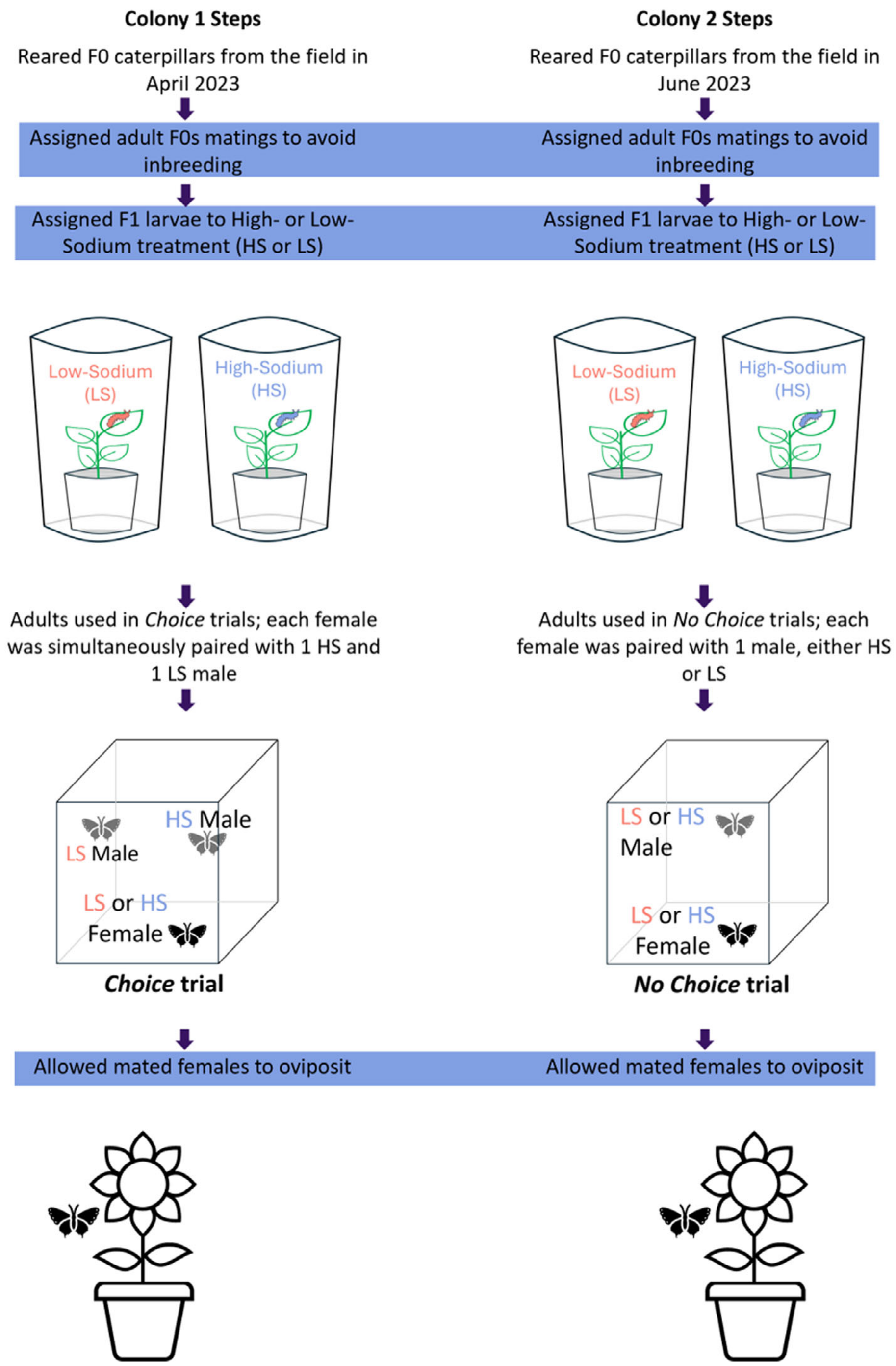

FIGURE 1 Experimental design from when larvae were collected in the field through their experimental trials. Colony 1 and Colony 2 followed the same general methodology, with a few deviations. Shared steps are highlighted in light purple boxes. The variations from the shared steps are shown on the outer portions, with Colony 1 on the left and Colony 2 on the right. All individuals, both female and male, were used once and not in repeated trials in both the Choice and No Choice mating trials.

mated with populations from Colony 2. After mating, each female was placed in a cage with a common sunflower host plant as a potential oviposition substrate. These planned matings resulted in unique F1 egg-clutch lineages, each of which was given a letter ID (Table S1) so that we could ensure that siblings were not placed together for any of the mating trials. These egg clutches provided the F1 offspring used in the experimental trials.

The rearing of egg clutches differed between Colony 1 and Colony 2. For Colony 1, each clutch was kept in a mesh-covered glass jar with a piece of filter paper that we moistened daily. When a clutch hatched, we moved it into a larger caterpillar jar. For Colony 2, we left each egg clutch on the plant where the female originally oviposited because we learned from the Colony 1 clutches that we had difficulties maintaining optimal moisture levels in the jars. Once the Colony

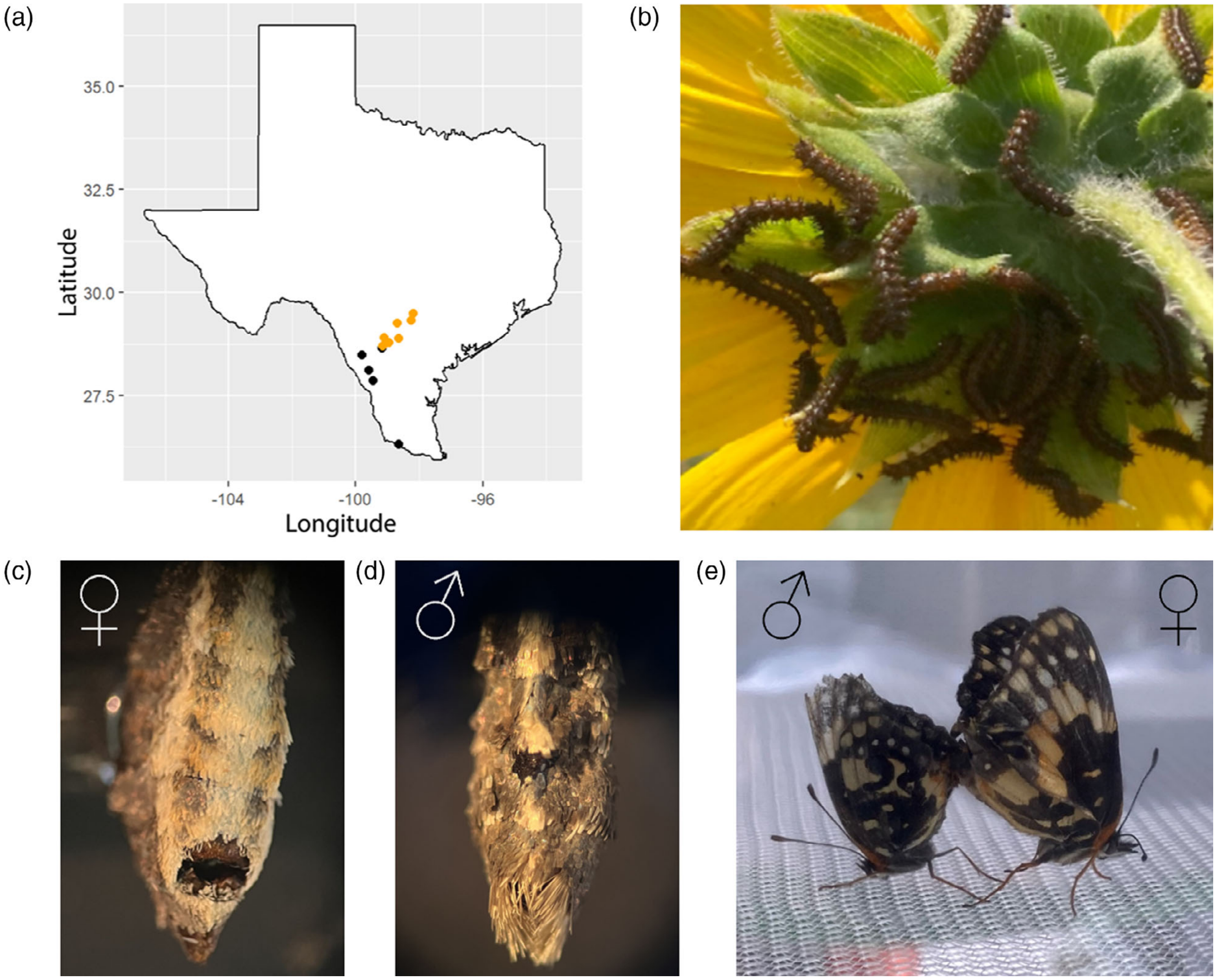

FIGURE 2 (a) Collection locations of *C. lacinia* Colonies 1 (black) and 2 (orange). Some populations came from the same collection location but were from different host plants several meters apart. (b) *C. lacinia* larvae on *Helianthus annuus*. (c) *C. lacinia* female genitalia under 20× magnification. (d) *C. lacinia* male genitalia under 20× magnification. (e) *C. lacinia* mating pair with the male on the left and the female on the right. The male is numbered on the left wing. Photos in B–E are by Maggie C. Vincent and the image in E is rotated clockwise 90° from the original.

2 clutches hatched, they were also moved into a larger caterpillar jar. These F1 individuals from both colonies were used in the sodium treatments so that we could control diets during most of their larval development.

Plant sodium treatments

To control larval sodium consumption, we grew plants in a hydroponic set-up to manipulate the sodium concentration in the leaf tissue. We potted seeds of domesticated common sunflower (*H. annuus*, Dwarf sunspot, sourced from Urban Farmer) and grew them in hydroponics following the protocol from Santiago-Rosario et al. (2023) using a Hoagland solution containing either no added sodium (0% added NaCl, i.e., low-sodium treatment) or 1.0% added NaCl (high-sodium treatment, 1 g Na per litre of solution). The Hoagland solution contained 1 M $Ca(NO_3)_2$, 1 M KNO_3, 1 M $NH_4H_2PO_4$, 1 M $MgSO_4*7H_20$, 9.2 mM $MnCl_2*4H_20$, 0.77 mM $ZnSO_4*7H_20$, 0.32 mM $CuSO_4*5H_20$, 0.11 mM MoO_3, 90 mM $FeSO_4*7H_20$ and 0.5 M H_2BO_3. We grew the plants with a light regime of 16 L:8 D, with a temperature of 23.3°C and a relative humidity of 60 ± 10%. We checked the plants daily and kept the water level constant. After 2 weeks, the old solution was removed, and a new Hoagland solution (and NaCl for the high-sodium treatment) was added to maintain the correct sodium concentrations for the plants. There were no overt differences in plant vigour between treatments, and plants grew well in both the low and high treatments. This methodology was based on Santiago-Rosario et al. (2023), wherein the same hydroponics set-up and sodium concentrations were used; low-sodium plant tissue had an average sodium concentration of 568 ± 215 ppm, and high-sodium plant tissues had 2296 ± 1451 ppm. We had insufficient hydroponics set-up space to also grow our oviposition plants, so we grew a separate set of plants in a greenhouse without added sodium for oviposition by test-subject butterflies during their behavioural trials. A set of

100 host plants was sown in nutrient-rich soil (Clegg's Nursery, Louisiana) in 2-L pots and kept in natural, well-watered conditions. We did not use any greenhouse plants for feeding; all feeding plants came from the hydroponic set-up. The greenhouse plants were only used for oviposition plants.

Larval sodium treatments

Prior to the 2nd or 3rd instar, individuals' small sizes made it difficult to move them without injuring or killing them (Santiago-Rosario et al., 2023). Once an F1 caterpillar reached the 2nd or 3rd instar, we placed it into an individual treatment of either low-sodium host plants or high-sodium plants (LS or HS, respectively) in an extruded polystyrene foam cup (17 cm height, 11.5 cm diameter) with a transparent lid and a hole in the lid's middle, covered with white mesh. Each cup contained a single caterpillar and a single host plant. Caterpillars were randomly assigned to treatments, and we alternated the placement of the treatment containers under the grow lights; every week the containers were randomly moved to avoid differences in the light conditions that could interact with or otherwise compromise our treatment differences. If a caterpillar consumed the entire host plant, we replaced it with a new host plant under the same sodium treatment and continued to do so until pupation. We did not measure total host plant consumption. We quantified when individuals pupated (if they survived to pupation), and how long individuals spent in the pupal stage. Once an individual pupated, we followed the pupal-rearing protocol detailed above. All individuals were maintained until they died. From Colony 1, we raised 100 individuals until adulthood (♀ LS $n = 29$, ♂ LS $n = 27$, ♀ HS $n = 17$, ♂ HS $n = 27$), and for Colony 2, we raised 65 individuals to adulthood (♀ LS $n = 19$, ♂ LS $n = 18$, ♀ HS $n = 14$, ♂ HS $\mathrm{n} = 14$). We measured the adult body size of a subset of Colony 1 individuals by measuring the right forewing in ImageJ (Schneider et al., 2012). Each forewing was measured from where the wing connects to the thorax to the wing's apex. The sample sizes for wing measurements were as follows: ♀ LS $n = 13$, ♂ LS $n = 24$, ♀ HS $n = 17$, ♂ HS $n = 16$.

Choice mating trials

The F1 adults from Colony 1 were used for the Choice mating trials, in which we avoided intra-lineage matings (and chose males matched for size—see below), but otherwise randomly paired a single LS or HS female simultaneously with both a LS and HS male. We held the Choice trials in June 2023, where a single female had equal access to one male of each treatment and was tested only once (15 LS and 9 HS females, for a total of $n = 24$ female-choice trials). For a single trial, we placed a LS and HS male in an experimental cage (30.5 × 30.5 × 30.5 cm, made of vinyl screening and polyester mesh; RestCloud Co.) and the female in a separate cage, and we left the cages outdoors in shaded areas overnight. For each experimental cage, we chose males of similar size, and we did not observe any male–male aggression between males placed in the same cage. The next day, we moved the cages into the sun for 30–40 min before starting the trials between 8:30 AM and 9:00 AM. The average daytime temperature was 29.2°C and the average night-time temperature was 23.4°C (Climate Report, 2023a). Once we added a female to an experimental cage (containing one of each type of male), we started timing. We counted male abdominal bends toward the female in the first 10 min, the latency to mate and the duration of copulation (see *Choice mating trials* section for details). Abdominal bends occurred when a male flanked a female closely (their abdomens within a few millimetres) and he bent his abdomen dramatically (we counted it as a bend if it was at least 45°) toward the female's abdomen. We recorded the identity of the male that succeeded in copulating, the time from the start of the trial until a pair started copulating (latency to mate, measured in minutes and seconds) and the duration of copulation (minutes and seconds). The start of mating was determined as the point when a male and female were joined and faced away from each other with their wings folded (e.g., see Figure 2e). We marked the end of copulation as the time when a male and female broke genital contact. We did not blind the observer to the identities of the male and female sodium treatments but rather relied on clearly distinguishable variables (e.g., connected vs. not connected), with little risk of informed observer bias. Additionally, male identity was often not determined until copulation started (i.e., imposed observer blindness) because it was less intrusive to read the male's ID at that point. We ran all trials for 90 min, but if a pair was copulating past the 90-min mark, they were left to complete copulation. Females were mated once, and after a completed copulation, we moved the female into a larger oviposition cage (40 × 40 × 60 cm, RestCloud Co.) with one sunflower host plant grown in the greenhouse (not in hydroponics, see above). Mated females were given a 20% sugar solution, which was replenished every day, and kept in these cages for oviposition until natural death (date recorded when possible). We checked for new egg clutches daily and uniquely numbered each new clutch. We did not reuse trial males (48 males total).

No Choice mating trials

The F1 adults from Colony 2 were used for the No Choice mating trials. We held the No Choice trials in August 2023. In the No Choice trials, a single female had access to a single male (♀ LS × ♂ LS = 2, ♀ LS × ♂ HS = 6, ♀ HS × ♂ LS = 3 and ♀ HS × ♂ HS = 3, for a total of $n = 14$ female-no-choice trials). Females and males were chosen to avoid intra-lineage matings, but otherwise at random from the appropriate sodium treatments. For a given trial, we kept the male and female in separate butterfly cages and left them outside overnight in shaded locations to acclimate. We moved the cages the next day into place in partially sunny locations 30–40 min before starting the trials (average daytime temperature = 32.3°C; average night-time temperature = 25.9°C; Climate Report, 2023b). We started timing a trial once the female was added to the single-male experimental cage. We counted abdominal bends that occurred in the first 10 min of the trial. Trials lasted 90 min, but pairs still copulating at the 90-min mark were left to complete the copulation. After a completed copulation, females were moved to an oviposition cage (40 × 40 × 60 cm, Rest-Cloud Co.) with a sunflower plant sourced from the greenhouse and kept as in the Choice trials. Trial males ($n = 14$) and trial females were only tested once and were not reused.

Reproductive output

We used the number of clutches, the number of larvae hatched and the number of larvae surviving to adulthood of the F2 generation as fitness proxies in the Choice trials, but not in the No Choice trials due to few occurrences of oviposition. The number of larvae hatched was determined by photographing a freshly hatched clutch and then enlarging the image (Surface Pro 7) to count the number of larvae. We maintained the F2 caterpillars in the same manner as the colonies, keeping them in jars and feeding them *H. annuus* or *A. trifida* leaves ad libitum. Each pupa was kept in a separate container until eclosion, and all eclosed F2 adults were frozen. We counted the number of pupae that successfully eclosed to determine the total number of adult offspring for each mated experimental F1 pair.

Statistical analyses

To test whether the Choice trials' F1 individuals differed in their body sizes depending on the sodium treatment they were fed as larvae, we ran a linear model including sex, treatment and their interaction as independent variables for forewing length as the dependent variable, a commonly used metric of body size in lepidopterans (García-Barros, 2000). We used the *lm()* function from the 'stats' package (R Core Team, 2024). Also, we used the 'lmtest' package (Zeileis & Hothorn, 2002) to check the model for independence, homoscedasticity and distribution of the residuals; no assumptions were violated.

For the subset of F1 individuals with complete eclosion and death data (LS = 44, HS = 37) from the Choice trials, we created survivorship curves using the 'survival' and 'survminer' packages (Kassambara et al., 2021; Therneau, 2024). Furthermore, we ran a generalised linear model (GLM) to evaluate whether sex, sodium treatment, or their interaction (independent variables) influenced survival (dependent variable). We checked the model residuals for independence, normality and homoscedasticity, and no violations were detected. The *glm()* function from the 'stats' package (R Core Team, 2024) was used for this analysis, and the package 'lmtest' (Zeileis & Hothorn, 2002) was used to check model assumptions.

We tested for a difference between the probability of mating between the Choice and No Choice trials by running a chi-square test. We used the *chisq.test()* function from the 'stats' package (R Core Team, 2024).

To analyse mating preferences in the Choice trials, we used a generalised linear mixed model (GLMM) to assess whether the dependent variable, female choice for a HS or LS male, was influenced by the independent variable, female's sodium treatment. The model employed a binomial distribution (HS = 1, LS = 0) and included female and male familial lineage as random effects to account for potential lineage differences, such as genetic differences. The analysis was conducted using the *glmmTMB()* function from the 'glmmTMB' package (Brooks et al., 2017). Model validation was performed with the 'DHARMa' package (Hartig & Hartig & Hartig, 2017) to check for overdispersion and detect potential outliers. We did not detect any overdispersion or outliers. We obtained statistical significance values using the *Anova()* function from the 'car' package (Fox & Weisberg, 2019). For mating preference, we also ran a chi-square test to assess whether females' mating choices (LS male, HS male or no mating) differed between larval treatments. We used the *chisq.test*() from the 'stats' package (R Core Team, 2024).

In the Choice trials, we used two GLMMs to evaluate the effects of female and male sodium treatments (independent variables), one for the latency to mate and the other for copulation duration (dependent variables). Both models included female and male familial lineages as random effects and used Gamma distributions for continuous data. Model residuals were simulated using the 'DHARMa' package (Hartig & Hartig, 2017) to check for dispersion and identify outliers. Neither model violated assumptions. We obtained statistical significance values as described above. For the latency to mate model, we performed a post hoc Tukey all-pairs comparison test using the *lmer()* function from the 'lme4' package and the *glht()* function from the 'multcomp' package (Bates et al., 2015; Hothorn et al., 2008). To determine whether the number of abdominal bends differed between LS and HS males, we ran a Friedman test using the 'stats' package (R Core Team, 2024).

For the reproductive outcomes of the Choice trials, we ran four GLMMs to evaluate the effects of female and male sodium treatments (independent variables) on the following dependent variables: number of clutches, number of hatched offspring, number of adult offspring and the average pupation time (in days). Female and male familial lineages were included as random effects in all four models. The model for the number of clutches used a Poisson distribution for count data, and model assumptions were verified as previously described. For the number of hatched offspring and the number of adult offspring, the initial Poisson models violated the assumptions. These were corrected by using a negative binomial distribution, and the revised models were validated and satisfied all assumptions. The model for average pupation time used a Gaussian distribution for continuous data. Model assumptions were tested, and none were violated. The same functions and packages were used for these analyses as described previously, and statistical significance values were obtained following the same approach.

Owing to small sample sizes, we did not test statistical significance in the No Choice trials for any of the mating preference or reproductive outcome variables. All statistical analyses were performed in R program Version 4.2.0 (R Core Team, 2024), and we used the 'ggplot2' package (Wickham, 2016) for visualisation.

RESULTS

Body size

Females in the Choice trials were 12% larger on average than males (♀ mean ± SE = 2.29 ± 0.02 cm, ♂ mean ± SE = 2.04 ± 0.02 cm). Moreover, forewing length was significantly influenced by sex, as indicated by our linear model ($F_{1,64} = 57.37$, $p < 0.001$, Figure 3, Table S2). By contrast, neither larval sodium treatment nor the interaction between sex and larval sodium treatment had a significant effect on forewing length ($F_{1,64} = 3.57$, $p = 0.06$; $F_{1,64} = 0.16$, $p = 0.69$, respectively, Figure 3, Table S2). On average, LS males had a forewing length of 2.02 ± 0.13 cm and HS males had a forewing length of 2.09 ± 0.12 cm. LS females had an average forewing length of 2.26 ± 0.13 cm, and HS females had an average forewing length of 2.31 ± 0.13 cm.

Adult lifespan

For the subset of Choice trial individuals with complete eclosion and death data, individuals raised on HS host plants survived an average of 6.8 days, while those raised on LS host plants lived 8.3 days, representing an 18.1% increase in lifespan (Figure 4, Table 1). High-sodium individuals were more likely to die during the first days of adulthood compared to LS individuals ($\chi^2 = 4.76$, $DF = 1, 77$, $p = 0.029$). Sex ($\chi^2 = 1.4$, $DF = 1, 77$, $p = 0.24$) and the interaction of sex and sodium treatment ($\chi^2 = 0.49$, $DF = 1, 77$, $p = 0.48$) did not significantly affect the survival probability. Lineage differences accounted for less than 0.012% of the variation across all models (Table S3).

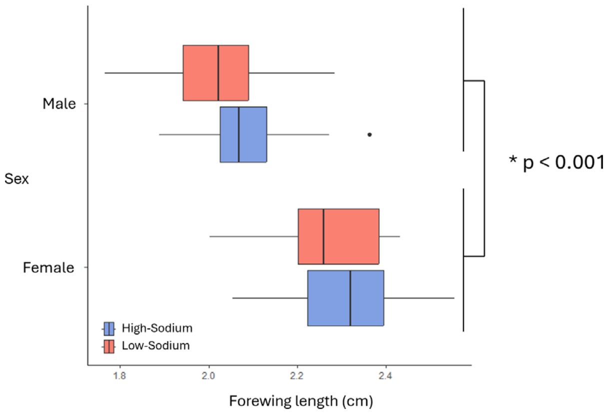

FIGURE 3 Forewing length as a proxy for body size in high-sodium (blue) and low-sodium (red) *C. lacinia* adult males and females. Females were significantly larger than males ($p < 0.001$), and no significant differences in body size were observed between individuals raised in high-sodium or low-sodium host plants. The boxes represent the interquartile ranges (IQR) with the median as the line within each box. The whiskers are 1.5 × IQR, and the black dots indicate outliers.

Mating probability in Choice versus No Choice mating trials

Mating occurred in only 36% of No Choice trials compared to 58% of the Choice trials, but this difference was not statistically significant ($\chi^2 = 1.14$, $DF = 1, 36$, $p = 0.29$). Due to the low mating success in No Choice trials, with sample sizes of only 1–2 per pairing combination (Table 2), we did not perform GLMM analyses for these trials. Additionally, there were no successful clutches from No Choice females, precluding any further analysis of reproductive output.

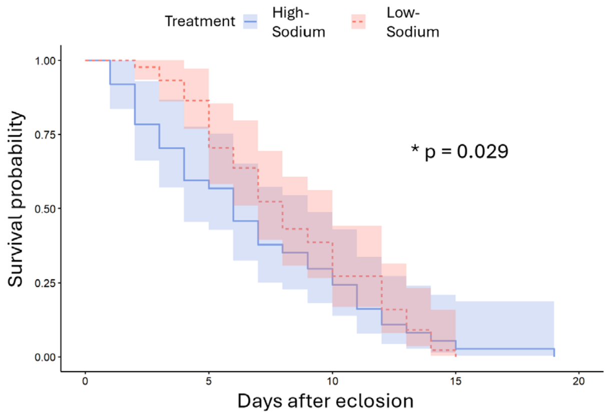

FIGURE 4 Survival curves for low-sodium versus high-sodium individuals. High-sodium individuals were more likely to die in the early days after eclosion and lived an average of 1.5 days shorter than low-sodium individuals ($p = 0.029$), except for one high-sodium individual that lived 19 days (blue tail on tight part of the figure). The shaded area for each survival curve represents the confidence interval.

TABLE 1 The average survival (in days) and the standard error for a subset of the Choice mating trials F1 individuals.

	♀ LS	♀ HS	♂ LS	♂ HS
Survival (days)	8.86 ± 0.77	6.92 ± 1.08	7.68 ± 0.75	6.75 ± 1.01
Sample size	22	13	22	24

Note: Means and standard errors are reported.
Abbreviations: HS, high sodium; LS, low sodium.

TABLE 2 Summary statistics of mating preferences and reproductive output for each of the parental treatment combinations from the No Choice mating trials.

	♀ LS × ♂ LS	♀ LS × ♂ HS	♀ HS × ♂ LS	♀ HS × ♂ HS
Mating success	2	1	1	1
Latency to mate (min)	76.75 ± 0.75	67	20	13
Copulation duration (min)	49.5 ± 2.5	58	50	47

Note: Means and standard errors are reported.
Abbreviations: HS, high sodium; LS, low sodium.

Mating preferences

Out of 24 Choice trials, 15 resulted in successful mating pairings. Among these, LS females mated with LS males in 35.7% of trials, with HS males in 28.6% of trials (Figure 5a), and did not mate in 35.7% of the trials. HS females mated with LS males in 50% of trials and HS males in 10% of trials (Figure 5a), and they did not mate in 40% of trials. Notably, there was only one successful pairing between a HS female and a HS male (Table 3). Female choice was not significantly influenced by male sodium treatment ($\chi^2 = 1.16$, $DF = 1, 13$, $p = 0.28$, Table S3), and female mating outcomes did not vary between sodium treatments ($\chi^2 = 2$, $DF = 1, 13$, $p = 0.32$). However, LS females had a longer average latency to mate than HS females (Table 3). Female sodium treatment significantly increased the latency to mate ($\chi^2 = 11.37$, $DF = 1, 13$, $p < 0.001$, Figure 5b, Table S3), while male sodium treatment had no effect (Figure 5b, Table S3). LS females paired with LS males exhibited a latency to mate three times higher than HS females paired with LS males (Tukey all-pair comparisons: $Z = 2.64$, $p = 0.04$, Figure 5b, Table S4). LS females had an average latency of 48.3 ± 12.4 min and 29.63 ± 10.2 min when they mated with LS and HS males, respectively (Table 3). HS females that mated with LS males had a latency of 15.8 ± 5.5 min, and the HS female × HS male pairing had a 0.5-min latency (Table 3). Copulation duration ranged from 29.5 to 45.5 min, with an overall average of 36.6 ± 5.6 min across all mating combinations (Table 3). There were no significant differences in copulation duration among pairing types, and neither female ($\chi^2 = 0.0003$, $DF = 1, 13$, $p = 0.99$) nor male treatment ($\chi^2 = 0.26$, $DF = 1, 13$, $p = 0.61$) influenced copulation duration (Figure 5c, Tables 2 and S3). The number of male abdominal bends was not influenced by the male's sodium treatment ($\chi^2 = 2$, DF = 1, 22, $p = 0.16$).

Reproductive output

Mated females in the Choice trials deposited between 1 and 5 egg clutches during their adult lifespans (Table 3). On average, 174 eggs hatched, and 77 larvae reached adulthood. Neither female nor male sodium treatment significantly affected the number of clutches ($\chi^2 = 0.05$, $DF = 1, 13$, $p = 0.82$; $\chi^2 = 0.05$, $DF = 1, 13$, $p = 0.82$; Figure 6a), the number of hatched offspring ($\chi^2 = 0.24$, $DF = 1, 12$, $p = 0.63$; $\chi^2 = 0.54$, $DF = 1, 12$, $p = 0.46$; Figure 6b), the number of adult offspring ($\chi^2 = 0.02$, $DF = 1, 13$, $p = 0.88$; $\chi^2 = 3.29$, $DF = 1, 13$, $p = 0.07$; Figure 6c), nor the average pupation duration of offspring ($\chi^2 = 1.05$, $DF = 1, 11$, $p = 0.3$; $\chi^2 = 1.27$, $DF = 1, 11$, $p = 0.26$; Figure 6d, Table S3).

DISCUSSION

An individual animal's physical condition can influence its mating behaviour, mating success, reproductive output and lifespan. A key determinant of physical condition, at any point in the life cycle, is the individual's diet during preceding developmental stages. Juvenile diet in insects may have long-lasting effects on adults' life-history traits

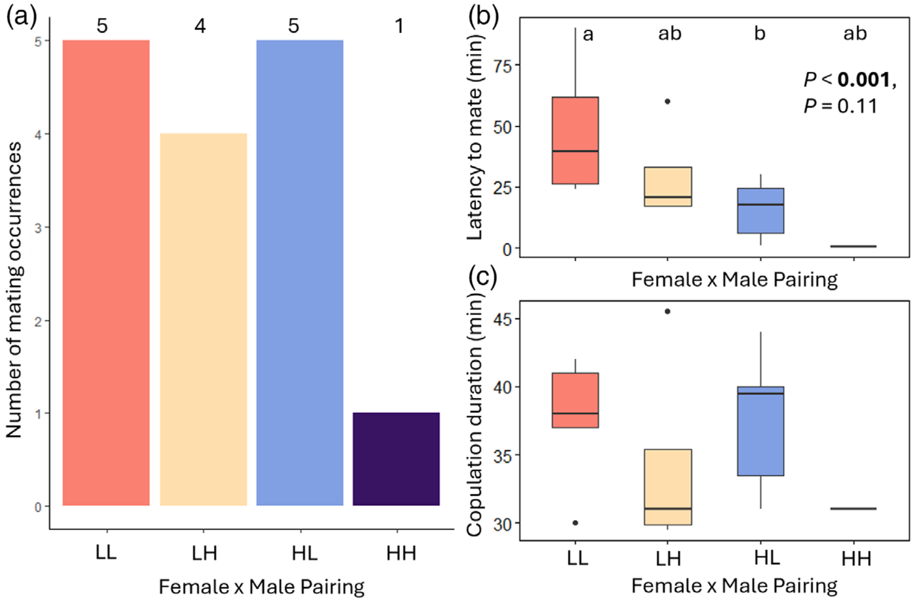

FIGURE 5 Mating preferences of Choice mating trials. (a) The number of matings that occurred for each female × male sodium treatment combination during the trials, (b) latency to mate (minutes), and (c) copulation duration (minutes) for each female × male treatment pairing. The *p*-value for female sodium treatment is listed first, followed by the *p*-value for male sodium treatment. The latency to mate was significantly longer for low-sodium females ($p < 0.001$) than for high-sodium females, while male sodium treatment had no effect. Female treatment is listed first: LL = Low-sodium female × Low-sodium male; LH = Low-sodium female × high-sodium male; HL = High-sodium female × Low-sodium male; HH = High-sodium female × High-sodium male. In (b) and (c), the black bars are the medians, the boxes are the interquartile ranges (IQR), and the whiskers are 1.5 × IQR. Outliers are represented with black dots.

TABLE 3 Summary statistics of mating preferences and reproductive output for each of the parental treatment combinations from the Choice mating trials.

	♀ LS × ♂ LS	♀ LS × ♂ HS	♀ HS × ♂ LS	♀ HS × ♂ HS
Mating preferences				
Mating success	5	4	5	1
Latency to mate (min)	48.3 ± 12.4	29.63 ± 10.2	15.8 ± 5.5	0.5
Copulation duration (min)	37.6 ± 2.1	34.3 ± 3.8	37.6 ± 2.4	31
Reproductive outcome				
Number of clutches	3 ± 0.82	2.5 ± 0.65	3 ± 1.15	3
Number of hatched offspring	202.5 ± 97.33	228 ± 87.37	162 ± 50.62	211
Number of adult offspring	107.5 ± 52.65	103.25 ± 40.76	17.33 ± 6.74	182
Average pupation time	6.17 ± 0.13	5.85 ± 0.26	5.82 ± 0.24	6.07

Note: Means and standard errors are reported.
Abbreviations: HS, high sodium; LS, low sodium.

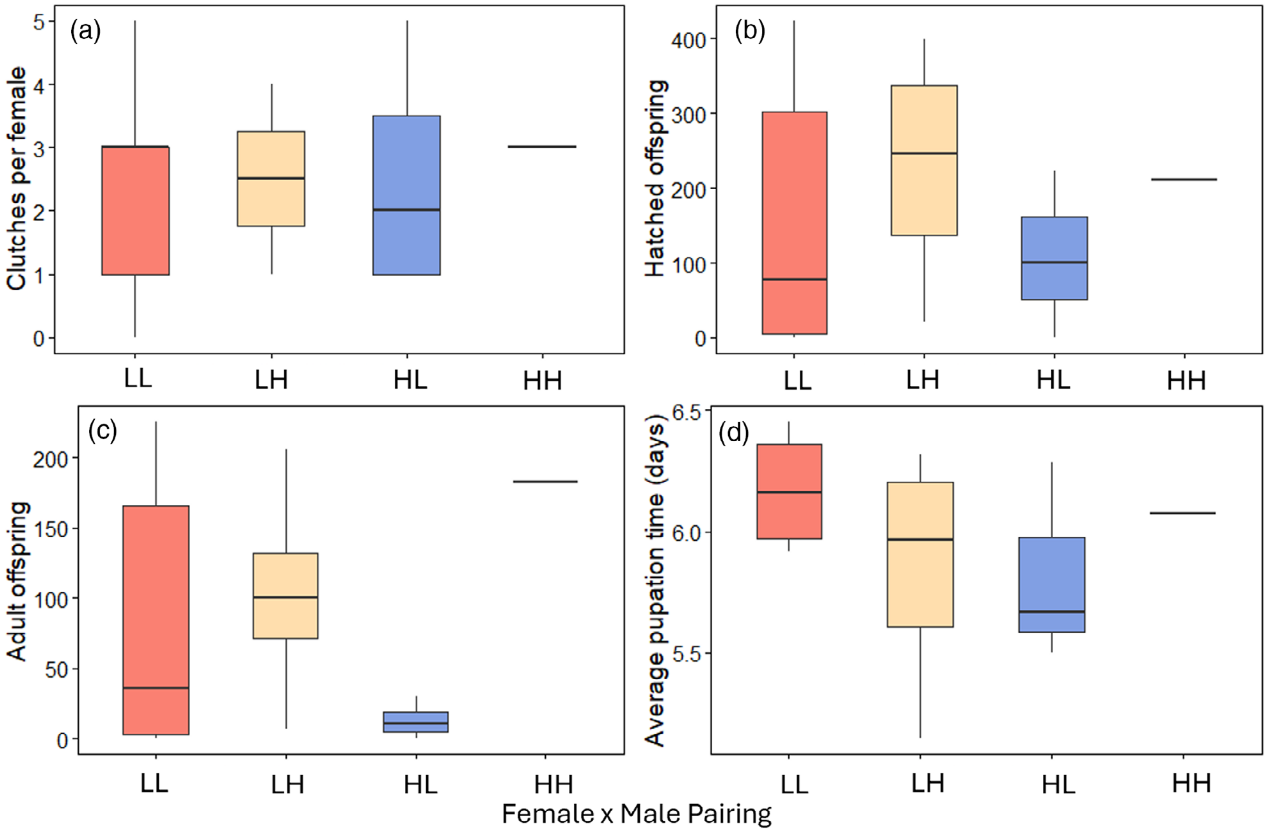

FIGURE 6 Reproductive outcomes from the Choice mating trials. Out of the 14 matings and resulting offspring, there were no significant differences in (a) number of clutches, (b) number of hatched offspring, (c) number of adult offspring, nor (d) the average pupation duration among any of the pairings. The *p*-value for female sodium treatment is listed first, followed by the *p*-value for male sodium treatment. Female treatment is listed first: LL = Low-sodium female × Low-sodium male; LH = Low-sodium female × High-sodium male; HL = High-sodium female × Low-sodium male; HH = High-sodium female × High-sodium male. The black bars are the medians, the boxes represent the interquartile ranges (IQRs), and the whiskers are 1.5 × IQR.

(Salgado & Saastamoinen, 2019) and lifelong reproductive success (Min et al., 2021). Previous work on lepidopterans, which develop through clearly defined ontogenetic niche shifts, has focused on the role of sodium acquisition during adulthood (Molleman, 2010; Pivnick & McNeill, 1987), but our experiments sought to understand how sodium availability during larval development may influence adult lifespan, reproductive behaviour and success. We predicted that HS individuals would have decreased lifespans and that males raised on

HS diets as larvae would have higher mating success than males raised on LS diets and that both females and males raised on HS diets would have higher reproductive output, with LS females being more choosy than HS females. We found support for our lifespan hypothesis, one of our mating preference hypotheses (latency to mate), but did not find support for our hypotheses regarding reproductive output. Knowing how insect herbivores respond to changing sodium regimes is key in a world where soil salinity is expected to increase in the coming decades (Palmgren & Shabala, 2024).

We found that HS individuals had reduced survival relative to LS individuals. In addition, for HS females, only one trial resulted in a HS female × HS male treatment pairing, suggesting a possible avoidance of this pairing. It is possible that sodium becomes detrimental at higher concentrations (Santiago-Rosario et al., 2023), resulting in avoidance by either or both of two HS opposite-sex individuals. High concentrations of sodium could decrease survivorship via mineral toxicity or oxidative stress (Calabrese & Baldwin, 2003). LS individuals lived an average of 1.5 days longer than HS individuals, a 22% increase. This difference in lifespan could mean that HS individuals have less time to find mates and, if female, find suitable oviposition sites. A time restriction could influence how individuals find and assess potential mates.

One key *C. lacinia* adult mating behaviour varied based on larval diets: the latency to mate was significantly longer when females mated with LS males. Even so, there was no difference in the length of copulations among sodium-diet pairings. Combined with the shorter lifespan in HS individuals, it could be that HS females adjust their pre-copulatory behaviour and are less choosy compared to LS females. Another possible explanation is that males differ in their behaviour prior to copulation such that LS males are less attractive to females or render females less receptive. In *Battus philenor* (L., 1771; Lepidoptera: Papilionidae), male flight behaviour varied depending on whether the adult males had access to sodium. Males given supplemental sodium had higher mating success than males without additional sodium, demonstrating that females may be able to discern differences in male condition prior to mating in at least some lepidopteran species (Mitra et al., 2016). It is unknown whether the difference in our results with *C. lacinia* compared to results from *B. philenor* represents a species-specific difference in the influence of sodium on behaviour or a difference in experimental details (e.g., our study tested the influence of larval diet, whereas the *B. philenor* project tested the influence of adult diet). There were no obvious differences in male behaviour of *C. lacinia* raised on different diets, but we did not explicitly quantify most aspects of displays (possibly courtship) prior to copulation. We only quantified male abdominal bends and that behaviour did not differ. Finally, while male body size did not differ significantly between sodium treatments, our *p*-value was marginal (0.06) and sample size was modest, suggesting a possible difference in size or other physical differences between individuals that we were unable to detect.

None of our fitness proxies varied among the different Choice trial treatment pairings. This finding does not support our prediction that reproductive output would be higher in the HS treatments than in the LS treatments but aligns with results from experiments with *B. anynana* (Molleman et al., 2004). Our fitness proxies were measures of indirect benefits to the females via their offspring and not to the females themselves. It could very well be that females benefit in other ways, for example, by the possibility of future matings, which could increase their reproductive success. To test this, we would need to measure rates of remating for both males and females. If males with higher sodium content are more likely to remate than lower sodium males, their total lifetime mating and reproductive success could be higher. If sodium is indeed a limiting resource for females, those that mate with lower sodium males may remain limited and may be more likely to seek additional matings. Additionally, we do not know how sodium may be transferred from males to females in *C. lacinia*. It could be via a spermatophore or through seminal fluids. Both males and females may have the option to exercise additional control over their success after any given copulation through post-copulatory behaviours.

We also found that females of *C. lacinia* were more likely to mate when they had more than one male present (Choice vs. No Choice mating trials). This could indicate a female preference for environments with multiple males or a facilitative mating effect of being able to exercise choice. Females may be primed or prefer to select between competing males. The Choice and No Choice trials were performed in June and August, respectively, so a difference in ambient temperature could alternatively explain the difference in mating proclivity between these two sets of trials. *C. lacinia* mates throughout August, but it is possible that the warmer ambient temperatures in August caused the overall lower inclinations to mate in the No Choice trials.

Based on our findings, *C. lacinia* may have a hormetic response to sodium, whereby sodium improves function up to an optimal dosage, but past that dosage results in adverse effects (Calabrese & Baldwin, 2003). Mineral toxicity in animals can result when organisms acquire too much of a mineral from their environment and risk upsetting homeostasis (Lall & Kaushik, 2021). High-sodium levels could also lead to oxidative stress and tissue damage. In mice, high-sodium levels led to an increase in reactive oxygen species and impaired organ function (Lu et al., 2017). These negative metabolic effects of sodium could create a trade-off between lifespan and reproduction (Flatt, 2011), which could explain the lack of a difference in reproductive success between LS and HS females. Our results suggest potential negative effects of high levels of sodium, but identifying an optimal amount of sodium was not an explicit goal of our experiments. More sodium treatments could identify a hump-shaped (hormetic) response to sodium that our binary treatments could not. Future work should examine development, performance and condition across a range of sodium availability throughout the life cycle of *C. lacinia*.

CONCLUSION

High-sodium availability decreased overall adult survival and decreased females' latency to mate. Pairings between HS females and HS males were especially infrequent, suggesting that too much sodium may have an adverse effect on female or male behaviour or physiology. Butterflies vary in terms of whether access to sodium increases reproductive success or not, but very little work has been done in this area. Understanding how sodium influences life history and behaviour in lepidopterans and other insects is crucial because soil salination, especially in agricultural fields and coastal areas due to oceanic salt intrusions, is expected to increase over the coming decades (Palmgren & Shabala, 2024). The number of drought events has increased globally over the last century, escalating the need for irrigation in agriculture (Liu et al., 2020). The water used in irrigation is increasingly saline, resulting in elevated soil salinity (Liu et al., 2020). It is key to understanding how changing soil salinity will affect multiple trophic levels, including insects that consume plants responding to drought and more saline environments. Lepidopteran larvae, in most cases, are less mobile than their adult counterparts, making them less able to seek out more favourable environments and shaping their life-history outcomes and fitness (Salgado & Saastamoinen, 2019). The sodium concentrations used in our experiment are well within the ranges that organisms may encounter in nature (Santiago-Rosario et al., 2022), meaning they may face even higher sodium concentrations in the future as drought causes plants to accumulate more sodium in tissues as an adaptive response (Ma et al., 2011; Sardans et al., 2008). Our results, as well as previous work in the European earwig (Wong & Kölliker, 2014), speckled cockroach (*Nauphoeta cinerea* [Burmeister, 1838]; Barrett et al., 2009), the Glanville fritillary butterfly (*Melitaea cinxia* [L., 1758], Salgado & Saastomoinen, 2019) and American bird grasshopper (*Schistocerca americana* [Drury, 1770]; Hahn, 2005), demonstrate that even for organisms undergoing hemi- or holometabolous development, the conditions of earlier life stages can affect adult condition. In the European earwig, juvenile dietary restriction resulted in females having smaller clutches despite adult females having the same access to food as adults (Wong & Kölliker, 2014). Ecological environment during larval development can result in deficits during adulthood that make individuals less competitive compared to those that came from a higher quality environment. Mating outcomes in adulthood may be influenced by circumstances during much earlier developmental stages, making it crucial to study all developmental stages to fully understand the determinants of lifetime reproductive success.

AUTHOR CONTRIBUTIONS

Maggie C. Vincent: Conceptualization; investigation; writing – original draft; methodology; visualization; software; formal analysis; data curation; validation; project administration. **Ana L. Salgado:** Conceptualization; investigation; methodology; validation; writing – review and editing; software; formal analysis; data curation. **Luis Y. Santiago-Rosario:** Conceptualization; funding acquisition; writing – review and editing; methodology; resources. **Kyle E. Harms:** Validation; writing – review and editing; methodology; supervision; resources.

ACKNOWLEDGEMENTS

We thank Jessica Eberhard, Claudia Husseneder and Morgan Kelly for their suggestions for the study and manuscript. We thank Stephen Midway for his statistical advice and Samantha Sanders for her help in caring for larvae during the experiment. We also thank the anonymous reviewers for improving this manuscript. This research was funded, in part, by the Texas Ecological Laboratory. LS-R is supported by the National Science Foundation (#2208922).

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

All data files and code are uploaded to Dryad and can be accessed with the following DOI: https://doi.org/10.5061/dryad.vx0k6dk2g.

ETHICS STATEMENT

The experiments represented in this manuscript did not require ethics approval.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

The authors did not reproduce materials from other sources in this manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Data S1. Supporting Information.

How to cite this article: Vincent, M.C., Salgado, A.L., Santiago-Rosario, L.Y. & Harms, K.E. (2025) Salty suitors: High larval sodium intake reduces adult lifespan and influences reproductive behaviour in a lepidopteran herbivore. *Ecological Entomology*, 1–13. Available from: https://doi.org/10.1111/een.70002