Received: 19 August 2019 | Revised: 8 November 2019 | Accepted: 14 November 2019

DOI: 10.1111/jeb.13574

RESEARCH PAPER

JOURNAL OF Evolutionary Biology ESEB WILEY

Extreme variation in testes size in an insect is linked to recent mating activity

E. V(Ginny) Greenway[1] | Lauren A. Cirino[1] | Daniela Wilner[1] | Ummat Somjee[1,2] | Maria-Eleni Anagnostou[3] | Russell T. Hepple[3] | Christine W. Miller[1]

[1]Entomology and Nematology Department, University of Florida, Gainesville, Florida

[2]Smithsonian Tropical Research Institute, Balboa, Ancon, Panama

[3]Department of Physical Therapy, University of Florida, Gainesville, Florida

Correspondence
E. V(Ginny) Greenway, Entomology and Nematology Department, Steinmetz Hall, Gainesville, FL 32608.
Email: egreenway@ufl.edu

Funding information
National Science Foundation, Grant/Award Number: IOS-1553100; University of Florida

Abstract

Ample sperm production is essential for successful male reproduction in many species. The amount of sperm a male can produce is typically constrained by the size of his testes, which can be energetically expensive to grow and maintain. Although the economics of ejaculate allocation has been the focus of much theoretical and empirical literature, relatively little attention has been paid to individual adult variation and plasticity at the source of sperm production, the testes themselves. We experimentally address this issue using the insect *Narnia femorata* Stål (Hemiptera: Coreidae). We established the metabolic cost of testicular tissue and then quantified variation in individual testes mass in response to multiple mate quality and quantity treatments. We uncovered extreme variation across individuals and considerable short-term effects of mating activity on testes dry mass. Importantly, the observed variation in testes mass was associated with notable fitness consequences; females paired with males with larger testes had greater hatching success. Overall, pairing with a female resulted in a 11% reduction in *dry* testes mass. Despite this apparent considerable mating investment, we found no evidence of strategic allocation to higher quality females or longer-term changes in testes mass. The dynamic nature of testes mass and its metabolic cost is vital to consider in the context of re-mating rates, polyandry benefits and general mating system dynamics both in this species and more broadly.

KEYWORDS

fertility, insect reproduction, mate choice, metabolic costs, sperm depletion, strategic allocation

1 | INTRODUCTION

The ability to produce gametes is crucial for reproductive success across all sexually reproducing species. In males, this gamete production takes place in a dedicated organ, the testes. Ultimately, although multiple factors are at play, testes size is likely to be the main physiological constraint on the amount of sperm a male can produce and the speed at which he can replenish his sperm stores after mating (Møller, 1988a, 1989; Vahed & Parker, 2012). As a result, testes size may impact a male's ability to engage in sperm competition, his fertilization success and his subsequent fitness. Thus, males should be under selection to maximize testes size. Despite the crucial role it can play in reproductive fitness, testes size is surprisingly variable across species (Byrne, Roberts, & Simmons, 2002; Møller, 1988b,

The peer review history for this article is available at https://publons.com/publon/10.1111/jeb.13574

1988a; Moller, 1989), within species (Awata, Heg, Munehara, & Kohda, 2006; Bailey, Gray, & Zuk, 2010), and even over time in the same individuals (Marie-Orleach et al., 2017). The reasons for this variation at the intraspecific and individual level are only beginning to be explored. Cues of high future sperm competition during development are known to frequently lead to increased investment in testes mass (Bailey et al., 2010; Johnson, Symonds, & Elgar, 2017). However, we know very little about how other factors surrounding mating activity influence the investment in testes.

At a fundamental level, the cost of developing and maintaining testicular tissue may limit testes size. Males may face a trade-off between maximizing investment in testicular tissue and functional fertility immediately, or conserving or diverting energy and resources towards future breeding opportunities or other physiological and life history traits (Sasson, Munoz, Gezan, & Miller, 2016; Simmons & Emlen, 2006; Somjee, Miller, Tatarnic, & Simmons, 2018). Massive reductions in testes mass outside of the breeding season provide evidence of the costs of maintaining testes. For instance, males of some species that breed seasonally undergo a 40%–90% reduction in testes mass when not reproductively active via testicular regression and recrudescence (Young & Nelson, 2001). We know little about why fluctuations in testes size occur rapidly (though see Marie-Orleach et al., 2017), over longer periods of time, or why in some cases fluctuations do not occur. Investigating factors that may influence testes mass is crucial for our understanding of the costs and benefits of sperm production, as well as the mechanisms regulating this process (Moore, 2014; Vasudev, Deeming, & Eady, 2014).

In this study, we started by establishing the metabolic cost of testes tissue in our study species and resolving the relationship between testes size, male body size and body condition. Our prediction was that testes should have a metabolic cost similar to other very costly tissues, and that larger males, in better condition, should have larger testes. We then examined the presumed benefit of increased investment in testes, by measuring the relationship between testes size and offspring production. We predicted that larger testes should lead to more offspring via a reduced likelihood that females will be sperm limited (Härdling, Gosden, & Aguilée, 2008; Joseph, Emberts, Sasson, & Miller, 2018; Wedell, Gage, & Parker, 2002).

With an understanding of the costs and benefits of testes investment, we next examined factors that may lead to increases or decreases in testes size. We predicted that exposure to mating opportunities, particularly with more females or females in better condition, would lead to an increase in male testes investment over time, which could enable greater fertilization success. We also predicted that males given the most recent mating opportunities would experience a temporary reduction in testes mass. We tested these predictions in the polyandrous insect *Narnia femorata* Stål (Hemiptera: Coreidae). *Narnia femorata* feeds on prickly pear cactus, the fruits of which males defend against rivals using their enlarged hind legs. Male testes mass is affected by diet quality and appears to trade off against investment in hind leg weaponry, providing prior evidence that testicular tissue is likely costly to both produce and maintain in this species (Joseph et al., 2018; Sasson et al., 2016). We raised males under laboratory conditions, measured the metabolic capacity of their testicular tissue and then quantified variation in their testes size across individuals of different ages and sizes. We manipulated male exposure to females of varying quality, quantity and novelty to assess if these factors were linked to differences in testes size. Our main objective was to provide one of the most extensive studies to date on how a range of both external social and intrinsic physiological factors can influence the growth and maintenance of the testes.

2 | MATERIALS AND METHODS

2.1 | General husbandry

All experimental *N. femorata* bugs were laboratory-reared individuals, the offspring of 29 parental pairs collected in Spring and Summer 2017 from Live Oak (30.26°N, −83.18°W) and Camp Blanding (29.95°N, −81.98°W) in North Central Florida. Clutches of nymphs produced by each parental pair were raised in plastic deli cups (top diameter 118 mm, bottom diameter 85 mm, height 148 mm) and provisioned with an *Opuntia mesacantha* ssp. *lata* cactus pad and ripe cactus fruit freshly collected between July and September from Camp Blanding. Nymphs were kept at densities of 5–13 bugs per deli cup at around 28°C under a 14:10 L:D cycle and checked daily. Once juvenile individuals reached fourth instar they were separated into individual deli cups, complete with cactus pad and late season cactus fruit, given a unique identifier and transferred to a temperature-controlled greenhouse. Upon eclosion to adulthood, all bugs were sexed and left to reach sexual maturity (14 days post-eclosion) with continuous access to ripe cactus fruit, with the exception of a subset of females in Part 2 for purposes of female quality manipulation (see below).

2.2 | Part 1: Quantifying metabolic activity in testicular tissue

To first assess levels of metabolic activity in testicular tissue, we quantified mitochondrial oxygen consumption in situ using permeabilized testes cells. This recently developed technique quantifies mitochondrial function whilst preserving the organelle's morphology and physiological interactions within cells and offers insights into mitochondrial respiratory function in vivo (Picard et al., 2011; Puurand et al., 2018). To our knowledge, this is the first application of the tissue permeabilization metabolic estimation method in an insect.

Briefly, the testes of 7 laboratory-reared *N. femorata* males were removed and the testicular tissue was permeabilized following Kuznetsov et al., (2008) (see Data S1 for further details) and weighed prior to respirometry trials, to calculate respiratory capacity per gram of testes tissue. To quantify tissue respirometry, the permeabilized testes tissue was placed within a respirometer in 2 ml of buffer B at 37°C and basal oxygen consumption was measured using a polarographic oxygen sensor housed in a

high-resolution respirometer (Oxygraph-2k, Oroboros, Innsbruck, Austria). Consecutive metabolic substrates from the electron transport chain were then added sequentially with a period of stabilization and measurement of O_2 flux between each step: 10 mM glutamate + 2 mM malate (GM; complex I substrates), 2 mM adenosine di-phosphate (ADP; to stimulate respiration), 10 mM succinate (SUCC; complex II substrate), 10 mM cytochrome c (Cyt C; to test for mitochondrial outer membrane damage during tissue processing), 10 mM antimycin A (AA; to inhibit complex III and thus stop normal electron flow to complex IV) and 5 mM ascorbate + 0.5 mM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD; artificial electron donor allowing estimation of complex IV flux capacity independent of upstream complexes). Following the same protocol, we also processed and measured the mitochondrial activity per gram of the hind leg weapon muscle tissue of another coreid, *Acanthocephala femorata*, for comparison.

2.3 | Part 2: Quantifying correlates of testes mass variation and effects of exposure to mating opportunities

271 sexually mature males were randomly assigned to one of three female companion treatments: (a) kept alone as a control, (b) paired with an unrelated virgin female raised on a cactus pad without fruit since adult eclosion or (c) paired with an unrelated virgin female raised on a cactus pad with a ripe fruit since adult eclosion. These dietary manipulations have been previously shown to considerably impact reproductive output in *N. femorata* (Joseph, Sasson, Allen, Somjee, & Miller, 2016; Sasson et al., 2016). In this case, female dietary manipulation successfully generated a considerable difference in fecundity between treatments: females given ad libitum access to cactus fruit produced approximately 70% more eggs over a subsequent month-long period of observation (mean 79.40 eggs ± 7.08 *SE* vs. fruit-deprived female mean 46.79 eggs ± 6.46 *SE*, Wilner, 2019).

Fruit-deprived females were provided with a ripe cactus fruit 24 hr prior to pairing with experimental males to limit any effects of food deprivation on mating receptivity. Pairs were then placed in the male's deli cup with a ripe fruit and a cactus pad planted in soil. To enable us to track changes in testes size and body condition over the 16-day experimental period, a randomly selected subset of males from each of the three treatments were euthanized every two days from the first day of pairing (i.e. on day 2 (n = 10–14), day 4 (n = 8–15), day 6 (n = 11–12), day 8 (n = 10–13), day 10 (n = 10–15), day 12 (n = 10–13), day 14 (n = 8–12) and day 16 (n = 11–13)) for subsequent measurement and dissection. All females were then left to oviposit for 32 days from the date of first pairing, and any eggs laid were scored for hatching success to quantify the effect of testes mass on reproductive success.

2.4 | Part 3: Quantifying effects of varying female quantity and novelty on testes mass

Insects were reared as described above, but with all individuals raised and kept as adults on a continuous high-quality diet of ripe cactus fruit and cactus pads. Males were randomly assigned to one of four treatments (see Figure 1b): (a) paired continuously with a different female every 48 hr (Novel Continuous), (b) paired continuously with the same female (Familiar Continuous), (c) paired for 3 hr every 4 days with the same female (Familiar Intermittent) or (d) paired with a different female for 3 hr every 4 days (Novel Intermittent). All individuals were between 14 and 21 days post adult eclosion upon initial pairing. Females used in Novel Continuous and Novel Intermittent treatments were switched between deli cups of males in the same

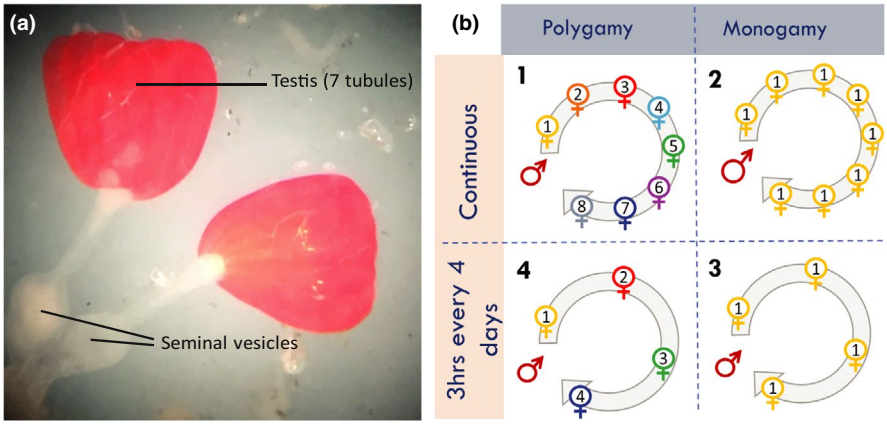

FIGURE 1 (a) *N. femorata* testes are each formed of 7 filaments or tubules in which sperm production occurs. These filaments are connected via the vas deferens to seminal vesicles, where sperm is stored prior to ejaculation (Photo credit: Paul Joseph) (b) A schematic of treatments used in Part 3 to manipulate the frequency and novelty of mating opportunities over a 16-day experimental period for focal. Treatments 1 (NC) and 4 (NI) utilized a round-robin design in which females were switched between focal males. For post hoc exploratory analysis, we combined treatments 1 and 2 (NC and FC, males given continuous access to either novel or familiar females) and treatments 3 and 4 (FI and NI, males given brief access every 4 days to novel or familiar females) to analyse the effect of time since last mating opportunity on testes mass

treatment in a round-robin design and thus were virgins in their first pairing but this was likely not the case in subsequent pairings. After 16 days of either continuous or intermittent pairing dependent on treatment, all bugs were euthanized and kept frozen at −18°C, prior to measuring and weighing.

2.5 | Quantifying body size and body mass

After freezing, all experimental males (and their female partners in Part 2) were imaged using a digital camera (Canon EOS 50D) and pronotum width was measured to the nearest micrometre using ImageJ software (v.1.46) (Schneider, Rasband, & Eliceiri, 2012). We then dissected out the testes of each male, removed their hind legs, placed these, along with the remaining body and fore- and mid-legs, in 70% EtOH and refrigerated prior to separating into preweighed aluminium foil boats. These were then dried for 72 hr at 60°C before being massed to the nearest microgram using a Mettler Toledo XP6 microbalance. Using this method, we obtained the dry mass of each male's testes, hind legs and remaining body (minus testes and hind legs). As seen in Figure 1a, *N. femorata* males possess paired testes, each consisting of 7 tubules or filaments at the tip of which spermatogenesis occurs (Moore, 2014). We measured testes dry mass in order to best quantify investment and changes in testicular somatic mass. All morphometric and dry mass data were collected blind to the male treatment group.

2.6 | Statistical analysis

All statistical analyses were performed in R v3.5.0 (R Core Team, 2018). For Part 1, we calculated the mean oxygen consumption rate across pooled tissue replicates in response to a series of substrates metabolized along the mitochondrial electron transport chain (see Data S1). In Part 2 analysis, body mass–testes mass static allometry was calculated using an ordinary least squares (OLS) regression between $\log_{10}$ transformed variables following Warton, Wright, Falster, and Westoby (2006), using the R package *smatr* to calculate slope and R^2 values for this scaling relationship (Warton, Duursma, Falster, & Taskinen, 2012). Male body condition was estimated using the scaled mass index (SMI) following Peig and Green (2009). Unlike other condition indices, SMI takes into account the scaling relationship between body mass and the linear size measurement (pronotum width in this study). SMI was calculated as $\hat{M}_i = M_i[L_0/L_i]^{b}\text{SMA}$ in which M_i is the body mass of individual i, L_i is the pronotum width of individual i, L_0 is the mean pronotum width, and ^{b}SMA is the scaling exponent calculated from a standardized major axis (SMA) regression of *ln*M on *ln*L.

We then quantified the effect of male testes mass on female reproductive success using a quasi-binomial GLM (due to overdispersion), with a two-column matrix comprised of the number of hatched eggs and unhatched eggs as the response variable (Demétrio, Hinde, & Moral, 2014). Females which did not lay eggs were not included in this step of the analysis. We focused on incomplete hatching success as a measure of female reproductive output as it is most likely to depend on male sperm production ability and reflects female reproductive constraint (Wedell et al., 2002). Female size, female diet treatment, male body mass, male testes mass and time spent paired with a male were all included as fixed effects. Using the same model structure and a negative binomial distribution, we also examined the effect of testes mass on the raw number of infertile eggs produced by each female (see Table S2).

To investigate determinants of male testes mass, body size, female companion treatment, body condition and treatment duration were included as fixed effects in a general linear mixed model (GLMM) framework using the *lme4* package, with testes mass (N = 271) as the dependent variable and family ID included as a random effect. Interactions between fixed terms were nonsignificant and therefore excluded from the final model. The significance of each term was assessed using a likelihood ratio test (LRT) between the full model and the same model minus the term of interest. Tukey's post hoc contrasts were performed using the *multcomp* package, with single-step adjusted P values (Hothorn et al., 2019). One individual was identified as an outlier, based on visual inspection of residual quantile plots and excluded from the analysis. This exclusion altered the significance of body condition and treatment duration as predictors of testes mass but the significance of all other terms remained qualitatively unchanged (see Table S1).

In Part 3, we included body size, treatment and body condition as fixed effects in an equivalent GLMM framework to the one used in Part 2, again with testes mass as the dependent variable and family ID as a random effect. Interaction terms were nonsignificant and, as in Part 2, subsequently excluded from the final model. We then conducted an exploratory analysis to tease apart the potentially independent effects of polygamy/ familiarity and time since last mating opportunity, by grouping treatments into two new variables as either polygamous (Novel Continuous and Novel Intermittent) or monogamous (Familiar Continuous and Familiar intermittent) and with either continuous access (Novel Continuous and Familiar Continuous) or a 4-day isolation period (Novel Intermittent and Familiar Intermittent) prior to euthanasia. The significance of each of these new variables was assessed, together with their interactions with body mass. Data are deposited on Dryad at https://doi.org/10.5061/dryad.x0k6djhfd.

3 | RESULTS

3.1 | Part 1—Quantifying metabolic activity in testicular tissue

We found that testicular tissue displayed somewhat lower levels of basal metabolic activity than costly hind leg muscle tissue but had a slightly higher maximum metabolic capacity. Permeabilized testicular tissue displayed a blunted level of mitochondrial respiratory activity in response to several metabolic substrates (GM, ADP, SUCC)

compared to hind leg weapon muscle tissue from a closely related coreid *Ancanthocephala femorata*. On the other hand, in *N. femorata* testicular tissue the flux capacity of mitochondrial Cytochrome c oxidase (complex IV of electron transport chain) appears to be similar or even higher than its *A. femorata* muscle equivalent, consuming 3.7 pmols of O_2 per second per gram of tissue versus 2.9 pmols of O_2 per second per gram of muscle tissue when stimulated with TMPD, an artificial electron donor (see Figure S1). The observed differences in response to metabolic substrates upstream of Cytochrome c oxidase between the two tissues suggest differences in stoichiometry in mitochondrial function along the respiratory chain between muscles and testes.

3.2 | Part 2—Quantifying correlates of testes mass variation and effects of exposure to mating opportunities

3.2.1 | Correlates of variation in testes mass

Male displayed an extremely high variance in testes dry mass; the heaviest testes dry mass measured was over 17-fold greater than the lightest (Min 0.042 mg, Max 0.722 mg), compared with a 10-fold difference between the highest and lowest male body masses recorded. Somewhat unsurprisingly, testes mass correlated significantly with body mass: larger males had larger testes (GLMM likelihood ratio test, $\chi^2_{(1)}$ = 192.44, p < .001, Figure S2). However, this scaling relationship, whilst positive, exhibited negative allometry (OLS regression, Slope = 0.88 ± 0.09 (95% confidence intervals), deviation from isometry r = −0.1682, p = .01) meaning larger males had proportionally smaller testes for their body mass than their smaller counterparts. Relative investment in testes mass ranged considerably, with testes comprising 4.1% of body mass in the largest instance down to only 0.4% of body mass in the smallest (mean relative testes mass = 1.6% of body mass). Males in better condition also had proportionally larger testes (GLMM LRT, $\chi^2_{(1)}$ = 4.055, p = .044). This variation in testicular mass impacted female reproductive success. Females paired with males with smaller testes produced relatively more unhatched eggs over a 32-day period (Figure 2, quasi-binomial GLM, LRT $\chi^2_{(1)}$= 7.96, p = .005, Table S2), when accounting for pairing duration, male size, female size and diet.

3.2.2 | Effect of exposure to mating opportunities on testes mass

Males paired with females had significantly smaller testes (12.9% lighter with fruit-fed females and 8.7% lighter with fruit-deprived females) than males kept alone (LMM LRT, $\chi^2_{(2)}$ = 16.363, p < .001, Figure 3). However, we found no evidence that the quality of the female a male was paired with was related to testes mass (post hoc Tukey's test, Z = −1.097, adjusted p = .516). To establish whether this decrease in mass upon mating opportunity exposure was limited to the testes or a more systemic body mass loss, we tested whether males paired with females had correspondingly lower body condition. This was not the case; we found no evidence that males differed in body condition across female companion treatments (GLMM, $F_{(2)}$ = 1.1545, p = .317).

Mean male testes mass (controlling for body size; Figure 4) fluctuated considerably over the course of the 16-day experimental period and was significantly influenced by the time point at which males were sampled (GLMM LRT, $\chi^2_{(7)}$ = 15.936, p = .026). However, there were no clear directional temporal effects over the course of the experiment and testes mass was neither significantly higher or lower in

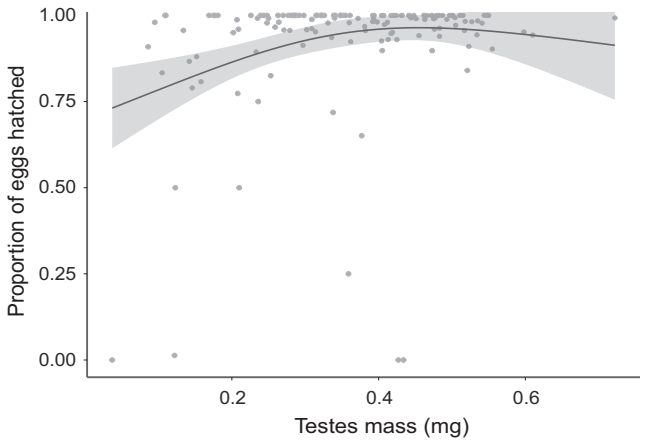

FIGURE 2 Male testes mass significantly affects female hatching success. Excluding individuals that failed to oviposit, females paired with males with larger testes produced relatively fewer unhatched eggs. The fitted line was produced using a basic general additive smoothing function (smoothing term set to k = 3) for visualization purposes only. Grey shading represents the 95% confidence region

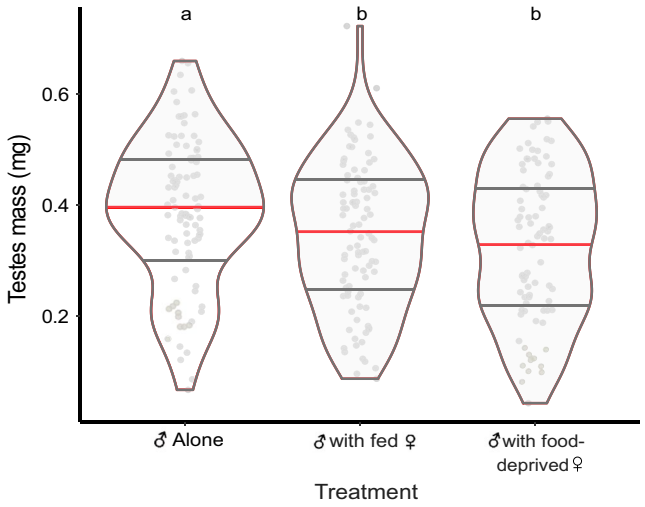

FIGURE 3 Effect of female companion treatment on male testes mass. Males kept alone (N = 94) had significantly larger testes than those paired with females fed a diet of either ripe fruit (N = 94) or deprived of fruit (N = 83). Bounds correspond to quartiles, with the red horizontal mid-line denoting the median. Letters denote statistical significance at the p < .05 level

males after 16 days versus those sampled after only 2 days (Post hoc Tukey's comparison, $Z = 1.272$, adjusted $p = .909$, see Figure 4). Thus, testes did not appear to increase in size as males aged.

3.3 | Part 3—Quantifying effects of varying female quantity and novelty on testes mass

Male testes mass was unrelated to the novelty of his female partner or the frequency of mating opportunities he was exposed to (see Figure S3, GLMM LRT, $\chi^2_{(3)} = 6.425$, $p = .093$). As in Part 2, body size accounted for the majority of variance in testes mass (GLMM LRT, $\chi^2_{(1)} = 152.89$, $df = 1$, $p < .001$), although in this experiment testes mass was not predicted by male body condition (GLMM LRT, $\chi^2_{(1)} = 1.345$, $p = .246$).

When treatments were pooled as described above (see Methods), we did however find a significant interaction between body size and time since last mating on testes mass (GLMM LRT, $\chi^2_{(1)} = 4.123$, $p = .042$, see Table 1, Figure 5). Larger males which were terminated immediately after a mating opportunity had reduced testes mass relative to individuals of the same size which had been separated from their mating partner for 4 days prior to termination.

4 | DISCUSSION

Here we demonstrate that testes mass is a highly dynamic trait, influenced not only by a male's size but also his body condition, exposure to mating opportunities and short-term mating history. Taken together, these factors generate extreme (17-fold) variation in adult testes dry mass as well as 10-fold variation in relative testes investment. These results contrast with historical assumptions and previous empirical evidence that primary reproductive trait development displays a degree of canalization in order to buffer fertility from environmental fluctuations (Eberhard et al., 1998; Emlen, Warren, Johns, Dworkin, & Lavine, 2012; Siegal & Bergman, 2002; Waddington, 1942). Furthermore, this observed testes mass variation had fitness consequences, influencing the reproductive output of a male's mating partner.

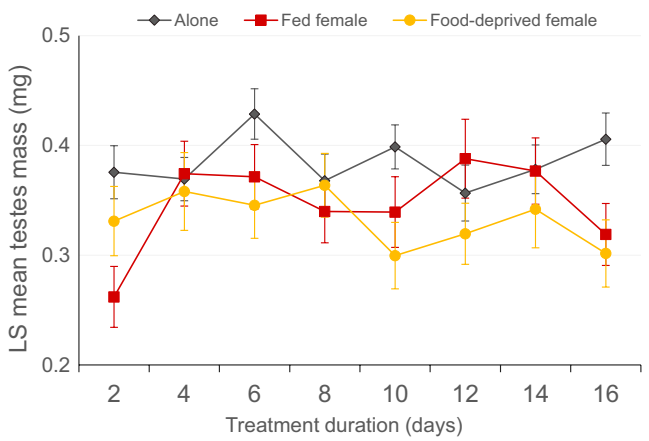

FIGURE 4 Effect of treatment duration on testes mass. Treatment duration (either time alone (grey), with a fed female (red) or a fruit-deprived female (yellow)) was significantly associated with variation in testes mass. However, there was no directional effect (i.e. either a significant increase or decrease) on testes mass over the course of the 16-day experimental period when controlling for body size. Post hoc Tukey's contrasts revealed a significant increase in testes mass between males sampled after 2 days and those in the treatment for 6 days (when controlling for body mass and companion treatment: $Z = 3.538$, adjusted $p = .01$). Bars represent ± 1 *SE*

The condition dependence of testes mass observed in this study, in addition to previous findings that testes size trades off with precopulatory traits (Joseph et al., 2018, 2016; Sasson et al., 2016), highlights that testicular tissue is costly to produce and maintain in this species. We confirmed the cost of this by quantifying mitochondrial activity in testicular tissue, finding that it appears to be capable of similar, if not higher, respiratory capacity as muscle tissue. This is consistent with the idea that gamete production in males is metabolically expensive and is likely limited by whole-body energetic constraints (Hayward & Gillooly, 2011; Somjee, Woods, Duell, & Miller, 2018). Despite this, we found no evidence that males limit testicular investment when deprived of mating opportunities. A potential explanation for this lies in the mating ecology of *N. femorata*. Given that these bugs' natural lifespans in Central Florida are unlikely to exceed 8 weeks (Cirino & Miller, 2017) and mate encounter rates in the wild may be unpredictable, the costs of maintaining comparatively large testes may be outweighed by the ability to capitalize on any mating opportunities as and when they arise. Although periods of exposure to mating opportunities increase allocation to sperm production in a range of longer lived vertebrate species (e.g. Beguelini, Góes, Rahal, Morielle-Versute, & Taboga, 2015; Cattelan & Pilastro, 2018; Olsson, Madsen, & Shine, 1997), shorter-term mating history appears to play a more significant role in determining testes mass in this invertebrate population over the 16-day period examined in this study. However tracking reproductive allocation over the course of each individual males' lifetimes may reveal that temporary fluctuations in testes mass form part of a longer-term sperm production optimization strategy in *N. femorata* as well.

In the short term, we found that males given access to mating opportunities display an 8%–12% decrease in *dry* mass. Simmons,

TABLE 1 Exploratory GLMM testing whether testes mass is associated with the separate effects of polygamy and time since mating, and their interaction with body mass

Fixed effect	X^2	*df*	*p* value
(Intercept)	0.453	1	.501
Body mass	270.442	1	**<.001**
Time since mating	0.907	1	.341
Polygamy opportunity	0.138	1	.711
Polygamy opportunity × body mass	0.094	1	.760
Time since mating × body mass	4.123	1	**.042**

p values <.05 are shown in bold.

Tomkins, and Alcock (2000) document a similar instance of testes wet mass reduction in mated versus unmated males in the Dawson's Burrowing Bee (−17% wet weight), which they ascribed to sperm transfer and subsequent depletion. Similarly, in Merino sheep, rams given access to mates for a period of 4–6 weeks had a 18%–26% decrease in scrotal volume, a metric which correlates tightly with testes wet mass (Knight, Gherardi, & Lindsay, 1987). The extent of this testes dry mass loss in *N. femorata* remains somewhat remarkable especially considering ejaculates are typically high in water content (Hopkins, Sepil, & Wigby, 2017). Although *N. femorata* males have seminal vesicles, organs typically used to store mature sperm prior to insemination, this interpretation of the change in testes mass suggests a considerable volume of sperm is also stored in the testes prior to transfer during copulation. This sperm depletion explanation is further supported by the finding from Part 3 (manipulating frequency of access to females), which revealed that time since last mating opportunity significantly influenced the scaling relationship between a male's body mass and his testes mass; males given 4 days to replenish sperm stores before freezing display a significantly steeper allometry. Interestingly, this effect was more pronounced in larger males, which tend to mate more frequently and potentially transfer larger ejaculates (Greenway, pers. obs), therefore requiring a longer time to replenish their sperm stores (e.g. Anthes, Werminghausen, & Lange, 2014). Confirming the source of this testes mass loss requires further histological and experimental investigation.

This variation in testes mass has consequences for a male's mating partners: females paired with males with larger testes had significantly higher hatching success. Although we cannot rule out the role of cryptic female choice, even under this noncompetitive scenario (Dougherty, Simmons, & Shuker, 2016), it appears that male sperm production has a role in constraining female offspring production. As a result, female choice for direct fertility benefits and assessment of male mating history may be promoted (Forbes, 2014; Wagner & Basolo, 2007). In guppies for instance, females avoid males observed consorting with other females enabling them to reduce their risks of sperm limitation (Scarponi, Chowdhury, & Godin, 2015). The fitness consequences of the considerable variance in testes mass for both mating partners merits further investigation, given the implications for mating system dynamics in *N. femorata*.

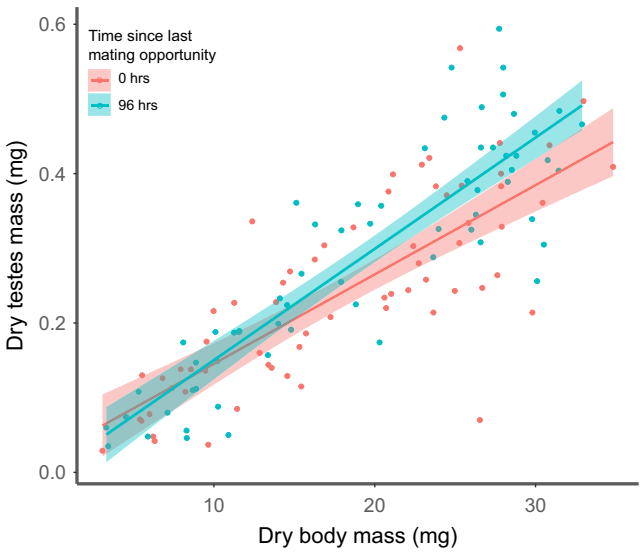

FIGURE 5 The relationship between body mass and testes mass is significantly influenced by the time since last mating opportunity. Males frozen immediately after a mating opportunity (red) had a significantly shallower scaling relationship between testes size and body mass than those given a 4-day period between their last mating opportunity and termination (blue) (GLMM LRT, $X^2 = 4.123$, $df = 1$, $p = .042$). Lines represent linear models with shaded 95% confidence intervals

Intriguingly, male sperm production investment appears to be independent of partner phenotype and familiarity, despite the considerable and possibly costly testes mass loss incurred through mating activity. Individuals paired with high fecundity females reared on ripe fruit did not differ in testes mass from those paired with low fecundity females (reared without fruit) over the two week experimental period. Given the mass loss incurred through mating activity, why do *N. femorata* males not tailor their reproductive investment to match their partner's fecundity? Firstly, males may be unable to detect differences in female quality, particularly given the fact that fruit-deprived females and fruit-fed females in this study did not differ in size because their diet was manipulated post-eclosion to their adult form, at which point their body size is fixed. A possible related explanation for this lack of strategic response to mate quality is the no-choice conditions under which the experiment took place. Males in Part 2 were only given access to one female (either fed or food deprived), potentially constraining the expression of any mating preferences that may become apparent if males are presented with multiple mating options simultaneously (Dougherty & Shuker, 2015).

Overall, our results caution against the common use of testes mass as a static metric of post-copulatory investment (as has historically been the case) and highlight the need to look beyond raw testes size to the detailed physiology of sperm production and testis architecture (Giannakara, Schärer, & Ramm, 2016; Moore, 2014; Ramm & Schärer, 2014; Schärer & Vizoso, 2007). We have demonstrated that, far from being static, testes mass can vary considerably according to an individual's mating history and is likely to fluctuate depending on the time since his last copulation. In addition, the 17-fold range in testes dry mass (vs. 10-fold range in body mass) observed in *N. femorata* highlights the importance of incorporating within-population variance, alongside the mean, when conducting interspecific comparisons. Although interspecific studies have provided, and continue to provide, a wealth of insights into reproductive investment and the strength of selection acting on sperm production (e.g. Byrne et al., 2002; Hosken, 1998; Kahrl, Johnson, & Cox, 2019; Lüpold, Linz, Rivers, Westneat, & Birkhead, 2009; Møller, 1988b; Stockley, Gage, Parker, & Møller, 1997; Vahed & Parker, 2012), there is still much to learn from within-species studies investigating the physiology of sperm production and its fundamental consequences for both male and female reproductive success.

ACKNOWLEDGMENTS

The authors wish to thank all students in 2018's ENY2890 CURE class for their role in collecting morphometric data for this project, as well as a team of dedicated undergraduate researchers, Emily Angelis, Liza Godfray, Madison Tharp, Haley Lenga, Ebony Taylor, Dani Gomez, Amun Majeed, Kathleen Wang and Elizette Rodriguez for their valuable assistance with insect rearing and data collection. We are also grateful to the Miller Lab for their insightful discussions and the feedback of two anonymous reviewers whose comments improved this manuscript. This study was supported by the National Science Foundation Grant IOS-1553100 to CWM and start-up funding provided by the University of Florida to RTH which made the mitochondrial function measurements possible.

ORCID

E. V(Ginny) Greenway https://orcid.org/0000-0003-0297-0927

REFERENCES

Anthes, N., Werminghausen, J., & Lange, R. (2014). Large donors transfer more sperm, but depletion is faster in a promiscuous hermaphrodite. *Behavioral Ecology and Sociobiology*, *68*(3), 477–483. https://doi.org/10.1007/s00265-013-1662-y

Awata, S., Heg, D., Munehara, H., & Kohda, M. (2006). Testis size depends on social status and the presence of male helpers in the cooperatively breeding cichlid *Julidochromis ornatus*. *Behavioral Ecology*, *17*(3), 372–379. https://doi.org/10.1093/beheco/arj043

Bailey, N. W., Gray, B., & Zuk, M. (2010). Acoustic experience shapes alternative mating tactics and reproductive investment in male field crickets. *Current Biology*, *20*(9), 845–849. https://doi.org/10.1016/j.cub.2010.02.063

Beguelini, M. R., Góes, R. M., Rahal, P., Morielle-Versute, E., & Taboga, S. R. (2015). Impact of the processes of testicular regression and recrudescence in the prostatic complex of the bat *Myotis nigricans* (Chiroptera: Vespertilionidae). *Journal of Morphology*, *276*(7), 721–732. https://doi.org/10.1002/jmor.20373

Byrne, P. G., Roberts, J. D., & Simmons, L. W. (2002). Sperm competition selects for increased testes mass in Australian frogs. *Journal of Evolutionary Biology*, *15*(3), 347–355. https://doi.org/10.1046/j.1420-9101.2002.00409.x

Cattelan, S., & Pilastro, A. (2018). Sperm priming response to perceived mating opportunities is reduced in male guppies with high baseline sperm production. *Current Zoology*, *64*(2), 205–211. https://doi.org/10.1093/cz/zoy008

Cirino, L. A., & Miller, C. W. (2017). Seasonal effects on the population, morphology and reproductive behavior of *Narnia femorata* (Hemiptera: Coreidae). *Insects*, *8*(1), 13. https://doi.org/10.3390/insects8010013

Demétrio, C. G. B., Hinde, J., & Moral, R. A. (2014). Models for overdispersed data in entomology. In C. P. Ferreira & W. A. C. Godoy (Eds.), *Ecological modelling applied to entomology* (pp. 219–259). Cham, Switzerland: Springer International Publishing. https://doi.org/10.1007/978-3-319-06877-0_9

Dougherty, L. R., & Shuker, D. M. (2015). The effect of experimental design on the measurement of mate choice: A meta-analysis. *Behavioral Ecology*, *26*(2), 311–319. https://doi.org/10.1093/beheco/aru125

Dougherty, L. R., Simmons, L. W., & Shuker, D. M. (2016). Postcopulatory sexual selection when a female mates once. *Animal Behaviour*, *116*, 13–16. https://doi.org/10.1016/j.anbehav.2016.03.003

Eberhard, W., Huber, B., Rodriguez, R. L., Briceno, R. D., Salas, I., & Rodriguez, V. (1998). One size fits all? Relationships between the size and degree of variation in genitalia and other body parts in twenty species of insects and spiders. *Evolution*, *52*(2), 415–431. https://doi.org/10.1111/j.1558-5646.1998.tb01642.x

Emlen, D. J., Warren, I. A., Johns, A., Dworkin, I., & Lavine, L. C. (2012). A mechanism of extreme growth and reliable signaling in sexually selected ornaments and weapons. *Science*, *337*(6096), 860–864. https://doi.org/10.1126/science.1224286

Forbes, S. (2014). Partial fertility and polyandry: A benefit of multiple mating hiding in plain sight? *Behavioral Ecology and Sociobiology*, *68*(8), 1329–1334. https://doi.org/10.1007/s00265-014-1743-6

Giannakara, A., Schärer, L., & Ramm, S. A. (2016). Sperm competition-induced plasticity in the speed of spermatogenesis. *BMC Evolutionary Biology*, *16*(1), 60. https://doi.org/10.1186/s12862-016-0629-9

Härdling, R., Gosden, T., & Aguilée, R. (2008). Male mating constraints affect mutual mate choice: Prudent male courting and sperm-limited females. *The American Naturalist*, *172*(2), 259–271. https://doi.org/10.1086/589452

Hayward, A., & Gillooly, J. F. (2011). The cost of sex: Quantifying energetic investment in gamete production by males and females. *PLoS ONE*, *6*(1), https://doi.org/10.1371/journal.pone.0016557

Hopkins, B. R., Sepil, I., & Wigby, S. (2017). Seminal fluid. *Current Biology*, *27*(11), R404–R405. https://doi.org/10.1016/j.cub.2017.03.063

Hosken, D. J. (1998). Testes mass in megachiropteran bats varies in accordance with sperm competition theory. *Behavioral Ecology and Sociobiology*, *44*(3), 169–177. https://doi.org/10.1007/s002650050529

Hothorn, T., Bretz, F., Westfall, P., Heiberger, R. M., Schuetzenmeister, A., & Scheibe, S. (2019). multcomp: Simultaneous Inference in General Parametric Models (Version 1.4-10). Retrieved from https://CRAN.R-project.org/package=multcomp.

Johnson, T. L., Symonds, M. R. E., & Elgar, M. A. (2017). Anticipatory flexibility: Larval population density in moths determines male investment in antennae, wings and testes. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1866), https://doi.org/10.1098/rspb.2017.2087

Joseph, P. N., Emberts, Z., Sasson, D. A., & Miller, C. W. (2018). Males that drop a sexually selected weapon grow larger testes. *Evolution*, *72*(1), 113–122. https://doi.org/10.1111/evo.13387

Joseph, P. N., Sasson, D. A., Allen, P. E., Somjee, U., & Miller, C. W. (2016). Adult nutrition, but not inbreeding, affects male primary sexual traits in the leaf-footed cactus bug *Narnia femorata* (Hemiptera: Coreidae). *Ecology and Evolution*, *6*(14), 4792–4799. https://doi.org/10.1002/ece3.2246

Kahrl, A. F., Johnson, M. A., & Cox, R. M. (2019). Rapid evolution of testis size relative to sperm morphology suggests that postcopulatory selection targets sperm number in Anolis lizards. *Journal of Evolutionary Biology*, *32*(4), 302–309. https://doi.org/10.1111/jeb.13414

Knight, T. W., Gherardi, S., & Lindsay, D. R. (1987). Effects of sexual stimulation on testicular size in the ram. *Animal Reproduction Science*, *13*(2), 105–115. https://doi.org/10.1016/0378-4320(87)90123-0

Kuznetsov, A. V., Veksler, V., Gellerich, F. N., Saks, V., Margreiter, R., & Kunz, W. S. (2008). Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. *Nature Protocols*, *3*(6), 965–976. https://doi.org/10.1038/nprot.2008.61

Lüpold, S., Linz, G. M., Rivers, J. W., Westneat, D. F., & Birkhead, T. R. (2009). Sperm competition selects beyond relative testes size in birds. *Evolution*, *63*(2), 391–402. https://doi.org/10.1111/j.1558-5646.2008.00571.x

Marie-Orleach, L., Vogt-Burri, N., Mouginot, P., Schlatter, A., Vizoso, D. B., Bailey, N. W., & Schärer, L. (2017). Indirect genetic effects and sexual conflicts: Partner genotype influences multiple morphological and behavioral reproductive traits in a flatworm. *Evolution*, *71*(5), 1232–1245. https://doi.org/10.1111/evo.13218

Møller, A. P. (1988a). Ejaculate quality, testes size and sperm competition in primates. *Journal of Human Evolution*, *17*(5), 479–488. https://doi.org/10.1016/0047-2484(88)90037-1

Møller, A. P. (1988b). Testes size, ejaculate quality and sperm competition in birds. *Biological Journal of the Linnean Society*, *33*(3), 273–283. https://doi.org/10.1111/j.1095-8312.1988.tb00812.x

Møller, A. P. (1989). Ejaculate quality, testes size and sperm production in mammals. *Functional Ecology*, *3*(1), 91–96. https://doi.org/10.2307/2389679

Moore, P. J. (2014). Reproductive physiology and behaviour. In D. M. Shuker & L. W. Simmons (Eds.), *The evolution of insect mating systems* (pp. 78–91). Oxford, UK: Oxford University Press.

Olsson, M., Madsen, T., & Shine, R. (1997). Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. *Proceedings of the Royal Society B: Biological Sciences*, *264*, 455–459.

Peig, J., & Green, A. J. (2009). New perspectives for estimating body condition from mass/length data: The scaled mass index as an alternative method. *Oikos*, *118*(12), 1883–1891. https://doi.org/10.1111/j.1600-0706.2009.17643.x

Picard, M., Taivassalo, T., Ritchie, D., Wright, K. J., Thomas, M. M., Romestaing, C., & Hepple, R. T. (2011). Mitochondrial structure and function are disrupted by standard isolation methods. *PLoS ONE*, *6*(3), e18317. https://doi.org/10.1371/journal.pone.0018317

Puurand, M., Tepp, K., Klepinin, A., Klepinina, L., Shevchuk, I., & Kaambre, T. (2018). Intracellular energy-transfer networks and high-resolution respirometry: A convenient approach for studying their function. *International Journal of Molecular Sciences*, *19*(10), 2933. https://doi.org/10.3390/ijms19102933

R Core Team (2018). *R: A Language and Environment for Statistical Computing*. Retrieved from http://www.r-project.org.

Ramm, S. A., & Schärer, L. (2014). The evolutionary ecology of testicular function: Size isn't everything. *Biological Reviews*, *89*(4), 874–888. https://doi.org/10.1111/brv.12084

Sasson, D. A., Munoz, P. R., Gezan, S. A., & Miller, C. W. (2016). Resource quality affects weapon and testis size and the ability of these traits to respond to selection in the leaf-footed cactus bug, *Narnia femorata*. *Ecology and Evolution*, *6*(7), 2098–2108. https://doi.org/10.1002/ece3.2017

Scarponi, V., Chowdhury, D., & Godin, J. J. (2015). Male mating history influences female mate choice in the trinidadian guppy (*Poecilia reticulata*). *Ethology*, *121*(11), 1091–1103. https://doi.org/10.1111/eth.12427

Schärer, L., & Vizoso, D. B. (2007). Phenotypic plasticity in sperm production rate: There's more to it than testis size. *Evolutionary Ecology*, *21*(3), 295–306. https://doi.org/10.1007/s10682-006-9101-4

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*, 671–675. https://doi.org/10.1038/nmeth.2089

Siegal, M. L., & Bergman, A. (2002). Waddington's canalization revisited: Developmental stability and evolution. *Proceedings of the National Academy of Sciences USA*, *99*(16), 10528–10532. https://doi.org/10.1073/pnas.102303999

Simmons, L. W., & Emlen, D. J. (2006). Evolutionary trade-off between weapons and testes. *Proceedings of the National Academy of Sciences USA*, *103*(44), 16346–16351. https://doi.org/10.1073/pnas.0603474103

Simmons, L. W., Tomkins, J. L., & Alcock, J. (2000). Can minor males of Dawson's burrowing bee, *Amegilla dawsoni* (Hymenoptera: Anthophorini) compensate for reduced access to virgin females through sperm competition? *Behavioral Ecology*, *11*(3), 319–325. https://doi.org/10.1093/beheco/11.3.319

Somjee, U., Miller, C. W., Tatarnic, N. J., & Simmons, L. W. (2018). Experimental manipulation reveals a trade-off between weapons and testes. *Journal of Evolutionary Biology*, *31*(1), 57–65. https://doi.org/10.1111/jeb.13193

Somjee, U., Woods, H. A., Duell, M., & Miller, C. W. (2018). The hidden cost of sexually selected traits: The metabolic expense of maintaining a sexually selected weapon. *Proceedings of the Royal Society B: Biological Sciences*, *285*(1891), 20181685. https://doi.org/10.1098/rspb.2018.1685

Stockley, P., Gage, M. J. G., Parker, G. A., & Møller, A. P. (1997). Sperm competition in fishes: The evolution of testis size and ejaculate characteristics. *The American Naturalist*, *149*(5), 933–954. https://doi.org/10.1086/286031

Vahed, K., & Parker, D. J. (2012). The evolution of large testes: Sperm competition or male mating rate? *Ethology*, *118*(2), 107–117. https://doi.org/10.1111/j.1439-0310.2011.01991.x

Vasudev, R., Deeming, D. C., & Eady, P. E. (2014). Developmental temperature affects the expression of ejaculatory traits and the outcome of sperm competition in *Callosobruchus maculatus*. *Journal of Evolutionary Biology*, *27*(9), 1811–1818.

Waddington, C. (1942). Canalization of development and the inheritance of acquired characters. *Nature*, *3811*, 563–565. https://doi.org/10.1038/150563a0

Wagner, W. E., & Basolo, A. L. (2007). The relative importance of different direct benefits in the mate choices of a field cricket. *Evolution*, *61*(3), 617–622. https://doi.org/10.1111/j.1558-5646.2007.00062.x

Warton, D. I., Duursma, R. A., Falster, D. S., & Taskinen, S. (2012). Smatr 3– an R package for estimation and inference about allometric lines. *Methods in Ecology and Evolution*, *3*(2), 257–259. https://doi.org/10.1111/j.2041-210X.2011.00153.x

Warton, D. I., Wright, I. J., Falster, D. S., & Westoby, M. (2006). Bivariate line-fitting methods for allometry. *Biological Reviews*, *81*(2), 259–291. https://doi.org/10.1017/S1464793106007007

Wedell, N., Gage, M., & Parker, G. (2002). Sperm competition, male prudence and sperm-limited females. *Trends in Ecology & Evolution*, *17*(7), 313–320. https://doi.org/10.1016/S0169-5347(02)02533-8

Wilner, D. (2019). Effects of nutritional stress during early adulthood on sexual behavior and long-term reproductive output in the leaf-footed cactus bug, *Narnia femorata* Stål (Hemiptera: Coreidae), Masters Thesis, University of Florida, USA.

Young, K. A., & Nelson, R. J. (2001). Mediation of seasonal testicular regression by apoptosis. *Reproduction*, *122*(5), 677–685. https://doi.org/10.1530/rep.0.1220677

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Greenway EV, Cirino LA, Wilner D, et al. Extreme variation in testes size in an insect is linked to recent mating activity. *J Evol Biol*. 2020;33:142–150. https://doi.org/10.1111/jeb.13574