The trade-off between investment in weapons and fertility is mediated through spermatogenesis in the leaf-footed cactus bug *Narnia femorata*

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Abstract

Males have the ability to compete for fertilizations through both pre-copulatory and post-copulatory intrasexual competition. Pre-copulatory competition has selected for large weapons and other adaptations to maximize access to females and mating opportunities while post-copulatory competition has resulted in ejaculate adaptations to maximize fertilization success. Negative associations between these strategies support the hypothesis that there is a trade-off between success at pre- and post-copulatory mating success. Recently, this trade-off has been demonstrated with experimental manipulation. Male leaf-footed cactus bugs, *Narnia femorata*, that lose a weapon by autotomy during development invest instead in large testes. While evolutionary outcomes of the trade-offs between pre- and post-copulatory strategies have been identified, less work has been done to identify proximate mechanisms by which the trade-off might occur, perhaps because the systems in which the trade-offs have been investigated are not ones that have the molecular tools required for exploring mechanism. Here we applied knowledge from a related model species for which we have developmental knowledge and molecular tools, the milkweed bug *Oncopeltus fasciatus*, to investigate the proximate mechanism by which autotomized *N. femorata* males developed larger testes. Autotomized males had evidence of a higher rate of transit amplification divisions in the spermatogonia, which would result in greater sperm numbers. Identification of mechanisms underlying a trade-off can help our understanding of the direction and constraints on evolutionary trajectories and thus the evolutionary potential under multiple forms of selection.

Introduction

Male-male competition has led to directional selection on weapon size (Andersson 1994, Emlen 2008). Increasing weapon size can lead to improved success in competition for territories and mating opportunities, but can be costly. Extreme weapon size may be countered by the increasing costs through natural selection (O'Brien et al. 2017). This leads to a potential trade-off between investing in weapons and other important fitness traits, including investment in fertility and other post-copulatory traits (Lüpold et al. 2014, Joseph et al. 2018, Somjee et al. 2018, Filice and Long 2018). Observational studies have demonstrated the predicted negative association between size of weapons and size of copulatory organs, including testes and ejaculates, supporting the idea of a trade-off between investment in traits leading to pre-copulatory and post-copulatory mating success. Males that lack weapons may compensate by investing more in any potential mating opportunity.

The trade-off between pre- and post-copulatory tactics has recently been experimentally tested in the leaf-footed cactus bug, *Narnia femorata* Stål (Hemiptera: Coreidae). Leaf-footed bugs use their hind limbs in contests with other males over the mating resource of a cactus fruit. Males that lose competition for territory may not disperse (Procter et al. 2012). Remaining on the cactus pad with the winning males and females means that males that lose pre-copulatory competition have the opportunity to compete for post-copulatory reproductive opportunities because they have access to females. Leaf-footed bugs are also able to autotomize (drop) their weaponized hind limbs when necessary (Emberts et al. 2016) and these lost limbs are not regenerated (Emberts et al. 2017). Males that have lost their hind limb weapon lose fights and are unable to establish dominance (Emberts et al. 2018), but can still mate as females will

accept copulations with weaponless males. Experimentally autotomized *N. femorata* males that have lost their weapon during development have reduced opportunity to invest in the weapons important for success in pre-copulatory competition. Instead, autotomized males reallocate resources into post-copulatory reproductive success by growing larger testes and have a fertilization advantage over intact males (Joseph et al. 2018), suggesting that autotomized males may be increasing sperm production.

While much work has focused on the selection and evolutionary outcome of the trade-off between pre- and post-copulatory success, much less work has been done on the mechanisms by which males might respond to an environment in which that trade-off occurs. Yet we know that both genetic and developmental processes can constrain or facilitate the evolution of traits (Maynard-Smith et al. 1985). Identifying these genetic and developmental mechanisms could be critical to understanding the targets of selection leading to the evolutionary trade-off (Zera and Harshman 2001). Mechanisms may be understudied in part because the biological systems in which these trade-offs are being studied are not easily dissected using modern tools, or their development has not been deeply characterized. *Narnia femorata*, however, belongs to the Hemiptera, a diverse group of bugs that have been widely studied and for which genomic resources are being developed (Panfilio and Angelini 2018). Here we take advantage of extensive information on testis development and spermatogenesis in a relative of *N. femorata*, the large milkweed bug, *Oncopeltus fasciatus*, to begin to identify the developmental mechanism by which autotomized males may be increasing sperm production that results in larger testis size.

Oncopeltus fasciatus is a hemipteran bug with a sequenced genome (Panfilio et al. 2019) that has been used as a physiological and developmental model system. Testis development (Economopoulos and Gordon 1971) and the process of spermatogenesis (Schmidt et al. 2002, Ewen-Campen et al. 2013) have been well documented. The testes of *N. femorata* and *O. fasciatus* have almost identical structures, consisting of 7 testis tubules enclosed within a pigmented membrane. The organization along the axis of each testis tubule is also extremely similar, with stages in spermatogenesis from the apical end of the *N. femorata* testis tubule easily recognizable based on our understanding of the progression of spermatogenesis in *O. fasciatus* (Figure 1A). We compared rates of cell division within the transit amplification divisions of the diploid spermatogonia in adult males who were either autotomized or intact. Our prediction was that we should see evidence of an increase in the transit amplification divisions within autotomized males, indicating an increase in the level of sperm production.

Methods

General husbandry

Adult *Narnia femorata* were collected from Starke, Florida (29.9803° N, 81.9848° W) and Live Oak, Florida (30.2642 N°, 83.1768° W) in the United States between 30 May and 20 June 2019. The second generation of offspring from these wild-caught bugs were raised in plastic cups (118 mm x 85 mm x 148 mm) with soil and a rooted *Opuntia mesacantha ssp. lata* cactus pad with ripe cactus fruits. Nymphs were kept at densities of 2-16 bugs per cup with temperatures between 24-28°C and 60-70% humidity. These cups were housed in a closed room under T5 HO fluorescent bulbs on a 14:10 L:D cycle and monitored every 48 hours to determine the date of 4^{th} instar emergence. In February 2020, within 10 days of becoming 4^{th}

instars and being assigned to treatment groups, the bugs were transported to The Moore Lab at the University of Georgia, Athens, United States. The cups of *N. femorata* were secured in plastic trays and transported in the back of a covered truck bed. Upon arrival at the Moore Lab, the bugs were placed in similar rearing conditions as in the Miller lab.

Experimental bugs

The impacts of autotomy at the onset of the penultimate stage on spermatogenesis were investigated as hemipterans are known to experience dramatic testes growth in these later stages of juvenile development (Economopoulos and Gordon 1971). Once nymphs reached the 4^{th} (penultimate) instar, they were randomly divided into one of two treatments: induced autotomy of the left hind limb or no autotomy (baseline control). Autotomy was induced by grasping the left hind limb by the base close to the body with forceps, allowing the bug to pull away and create a break at the joint between the trochanter and femur (Emberts et al. 2016). Once the treatments were applied, the 4^{th} instar nymphs were placed on cactus in groups of 3-4 siblings per cup as conspecific density can impact development. Within high concentrations of *N. femorata*, faster developing siblings have much larger body sizes compared to the last siblings to develop into adults, which suggests strong competition between siblings (Allen and Miller 2020). By placing nymphs in groups of 3-4, the impacts of conspecific density on development were minimized and consistent for the groups. Within 48 hours of becoming adults, the male bugs were separated into their own individual cups with a cactus pad and fruit.

Dissection and staining of testes

Between 21- and 28-days post adult emergence, the testes were removed from males. Individual tubules were separated from the outer membrane for fixation and staining. Testis

tubules from males within a treatment were pooled for staining if they were dissected on the same day. The testis tubules were fixed in 4% formaldehyde in PBS plus 0.1% Triton X-100 (PBT) for 30 minutes. The tubules were stained with α-phosphohistone H3 Ser10 (pHH3) primary antibody (Millipore antibody 06-570, Sigma-Aldrich, St. Louis, MO). α-pHH3 stains for chromosome condensation in preparation for mitosis and meiosis (Hans & Dimitrov 2001, Prigent & Dimitrov 2003). The secondary antibody was an Alexa Fluor goat-anti-rabbit 647 (ThermoFisher Scientific, Waltham, MA). Following antibody staining the tubules were stained with DAPI (0.5 μg/mL PBT) to visualize nucleic acids. Stained tubules were mounted in Mowiol 4-88 mounting medium (Sigma-Aldrich, St. Louis, MO). Slides were kept in boxes to limit light exposure and stored at 4°C until visualized. The testis tubules were visualized with a Zeiss LSM 710 Confocal Microscope (Zeiss) at the UGA Biomedical Microscopy Core Facility. All testis tubules were photographed and included in the analysis.

Analysis of division rates within spermatogonia and spermatocytes

To test the prediction that autotomized males would show higher division rates, estimated from the number spermatocysts positively stained with α- pHH3, than intact males, the number of spermatocysts stained with the α-pHH3 antibody was scored in the photographs of testis tubules from males in the two treatments. The images were divided into the two regions and stained spermatocysts were counted separately for Region 1, containing spermatogonia undergoing mitotic transit amplification divisions and Region 2, containing spermatocytes undergoing meiotic division (Figure 1B). Only positively stained spermatocysts were counted. Single cells were occasionally stained, perhaps representing endoreplication within the cyst cells that enclose the spermatocysts (Figure 1B). These were not included in the analysis. Prior

to analysis, the photographs were coded by an independent observer to allow for the data to be collected blind with respect to treatment. To check for interoperate error, two people counted a subset of images for stained spermatogia in Region 1 and spermatocytes in Region 2. There was good agreement between the two sets of data indicating that the counts were reliable. While the data was count data, our results included sufficient numbers of categories to allow for the data to be analyzed by ANOVA using JMP Pro version 14.

Results

Regions of spermatogenesis were easily identifiable within the testis tubule (Figure 1A) based on our understanding of spermatogenesis in *O. fasciatus* testis tubules (Ewen- Campen et al. 2013, Washington et al. 2020). At the apical tip of the testis tubule there were spermatocysts containing spermatogonia. Spermatogonia divide mitotically and are recognizable by their relatively dense, uniformly stained nuclei. Posterior to the spermatogonia are the spermatocytes that undergo meiosis to form the haploid spermatids. Spermatids undergo differentiation to form spermatozoa.

Spermatocysts in the most apical region of the testis tubule (Figure 1B; sg) were more likely to be stained with α- pHH3 antibody than spermatocysts in the region below (Figure 1; sc) where spermatocytes are undergoing meiosis ($F = 34.723$, d.f. = 1, 63, $p < 0.001$), indicating that rates of cell division were greater in the spermatogonia than spermatocytes, as expected. Among spermatogonia undergoing transit amplification divisions, autotomized males were more likely to have spermatogonial spermatocysts stained with α- pHH3 than control males (Figure 2A; $F = 7.034$, d.f. = 1, 35, $p = 0.012$). Among spermatocytes undergoing meiosis, there was no

151 difference in the number of spermatocysts stained with α- pHH3 (Figure 2B; F = 0.479, d.f. = 1,
152 35, p = 0.494).

153 **Discussion**

154 While many studies have investigated the evolutionary outcomes of the trade-off among pre-
155 and post-copulatory strategies, fewer have investigated the mechanisms by which that trade-
156 off is mediated. We took advantage of the wealth of knowledge about a related model insect,
157 *O. fasciatus*, to explore how loss of a weapon during testis development impacted
158 spermatogenesis in *N. femorata*. We found that the increase in testis size and fertilization
159 advantage in autotomized male *N. femora*ta was associated with an increased rate of mitotic
160 divisions in spermatogonia. Plasticity in sperm numbers and quality under variable conditions
161 has been explored (Moatt et al. 2014, Bunning et al. 2015, Dávila & Aron 2017, Joseph et al.
162 2016, Somjee et al. 2018), but few have examined the mechanism by which the increase in
163 sperm numbers occurred. In *Drosophila melanogaster*, males respond to perceived sperm
164 competition risk by increasing sperm production (Moatt et al. 2014). A recent study has shown
165 that mating increases the division rate of germline stem cells in the testes of *D. melanogaster*
166 through G-protein signaling (Malpe et al. 2020). However, this sort of mechanistic studies
167 depends on established molecular markers to identify the germline stem cells and niche cells as
168 well as genetic tools lacking in most species.

169 In *O. fasciatus*, and presumably *N. femorata*, sperm arise originally from germline stem cells at
170 the tip of each testis tubule (Schmidt et al. 2002). As shown in *D. melanogaster* (Malpe et al.
171 2020), variation in sperm production could arise through the rate of production of germ cells
172 through division of the germline stem cells. Alternatively, variation could arise through the

modulation of the sperm production process (Kaczmarczyk and Kopp 2001, Extavour 2013, Moore 2014, Ramm & Schärer, 2014). While we do not have the tools to examine germline stem cell turnover in either *N. femorata* or *O. fasciatus*, we have been able to show an impact of losing a weapon during larval development on the rate of spermatogonial divisions in adults. The timing of autotomy corresponds with a critical period of testis development. In *O. fasciatus*, testes are small and undeveloped in the 1st through 3rd instars. During the 4th instar stage of development, meiosis is initiated and spermatids begin to form (Economopolous and Gordon 1971, Schmidt et al. 2002). If, as we predict, the developmental timing is the same in *N. femorata*, then autotomy during the 4th instar stage of development is a time when the testis could benefit from increased resource allocation, either in increasing the number of germline stem cells (Kaczmarczyk and Kopp 2001) or increasing the number of spermatogonia that enter the maturation pipeline (Moore 2014).

The trade-off between weapons critical for pre-copulatory mating success and traits critical for post-copulatory fertilization success, has now been demonstrated in a number of species. Males that lack weapons tend to have increased fertilization success (Joseph et al. 2018, Somjee et al. 2018, Van den Beuken et al. 2018). Males that invest in precopulatory traits may not be able to fully invest in post-copulatory traits (Parker and Pizzari 2010, Parker et al. 2013). The constraint on investment in these strategies could be genetic (Filice and Long 2018) or depend on resource allocation (Joseph et al. 2018, Somjee et al. 2018). While there is much to be discovered about molecular and physiological control of spermatogenesis in *N. femorata*, studies such as this will allow researchers to dissect prospective targets of selection at a molecular level, opening up the potential for more targeted experimental manipulation.

Ultimately, integrating the fitness outcomes of these trade-offs with the molecular and cellular control mechanisms will allow us to examine the way in which selection shapes, or is constrained by, mechanism.

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Conflicts of Interest: None declared.

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Data Accessibility: The datasets analyzed for this study will be made available by the authors through the publicly available Dryad Digital Repository (https://datadryad.org/stash/) upon acceptance of the manuscript.

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Figure Legends

Figure 1: Structure of *N. femorata* testis tubule. (A) A low magnification image (6X) of a *N. femorata* testis tubule stained with DAPI (DNA). The progression through spermatogenesis could be clearly differentiated based on nuclear morphology. In region 1 spermatocysts containing spermatogonia were identified based on the number of nuclei and nuclear morphology. Spermatogonia (sg) have dense, uniformly stained nuclei. They undergo mitotic transit amplification divisions to give rise to spermatocysts containing 64 nuclei in *O. fasciatus* (Ewen-Campen et al. 2013). In region 2 the spermatocytes (sc) undergo meiosis to form haploid spermatids (st) which then differentiate into spermatozoa (sz). (B) Higher magnification (12X) image of a representative testis tubule stained for both DNA (DAPI, cyan blue) and dividing cells (α- pHH3 antibody; magenta). In this image, within region 1 (demarcated with the yellow dotted line) 5 spermatocysts containing synchronously dividing spermatogonia were labeled (yellow arrows). Occasionally single nuclei were labeled with antibody (yellow star). These are likely to represent endoduplication of the cyst cell nuclei and were not included in the counts as they were clearly not spermatogonia or spermatocytes given that all nuclei within a cyst divide synchronously. In region 2 (demarcated with the white dotted line), spermatocysts at the boundery of the spermatocytes and spermatid are labeled with α- pHH3. While typically there are fewer spermatocysts dividing in this region, in this image there were 5 spermatocysts labeled as they progressed through meiosis, identified by the number of nuclei within the cyst (white arrows). Occasionally single cells were labeled (white star) in this region, again, presumably cyst cell nuclei.

328 **Figure 2**: The number of spermatocysts from control males and autotomized males of the leaf-
329 footed cactus bug, *Narnia femorata* that were positively stained with an α- pHH3 antibody. (A)
330 Spermatocysts in region 1 containing spermatogia from autotomized males were stained more
331 frequently than those in the testis tubules of control males. (B) There was no difference in the
332 number of spermatocysts in region 2 containing spermatocytes undergoing meiosis between
333 autotomized and control males. Black dots indicated the mean value for each treatment, gray
334 dots are individual data points. Each error bar is constructed using 1 standard error from the
335 mean
336

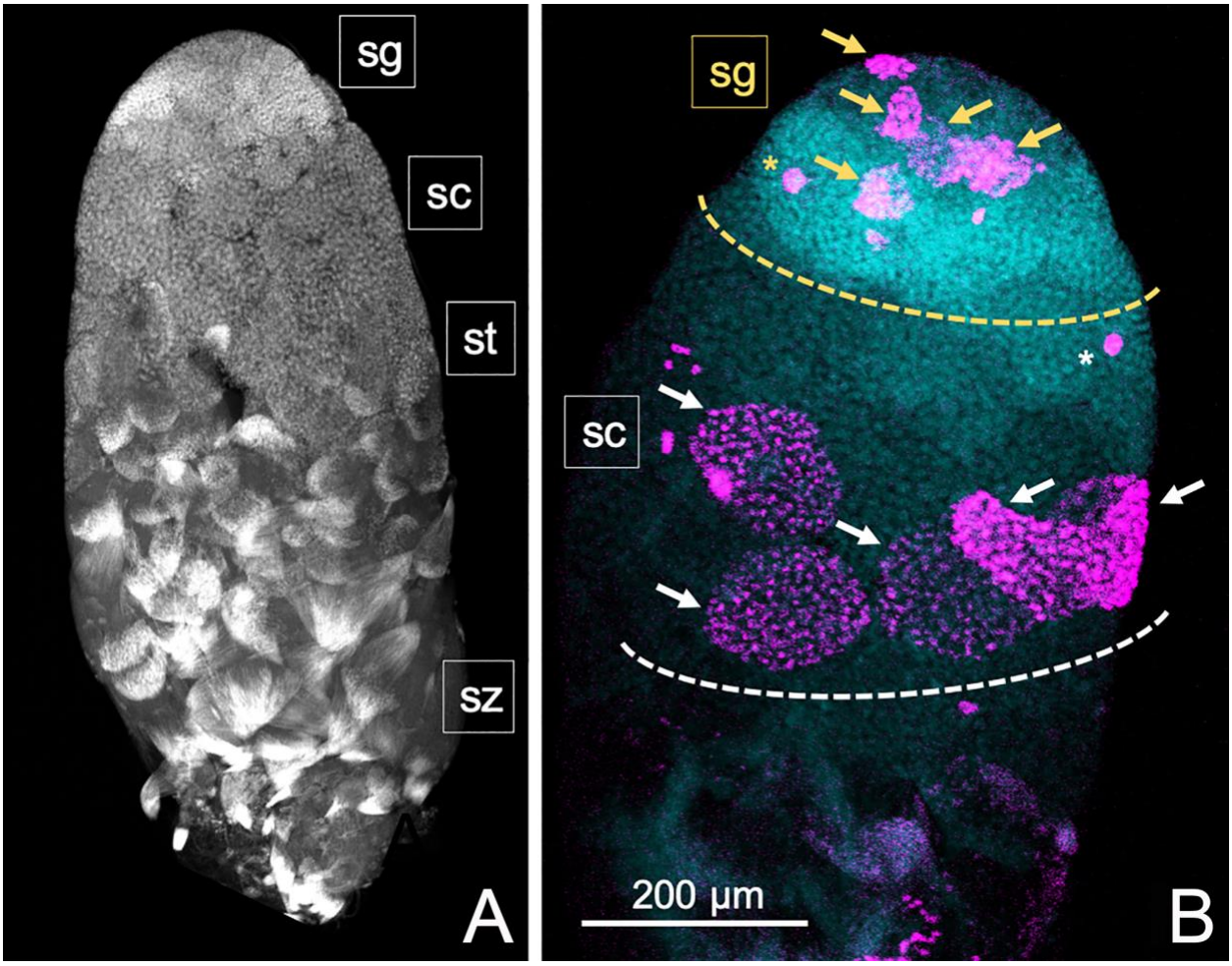
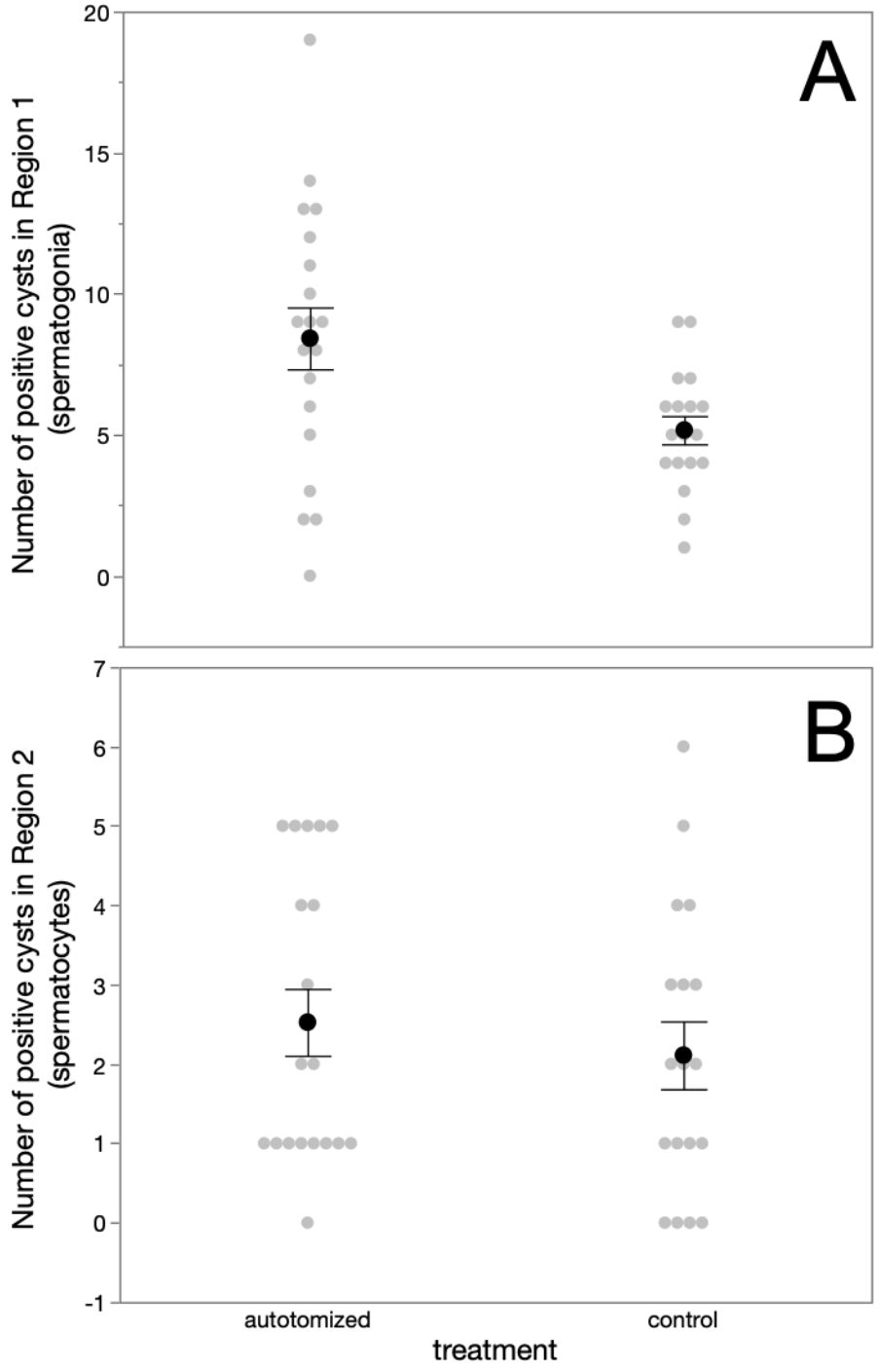


Figure 1.



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341 Figure 2.