Behavioral Ecology

The official journal of the
ISBE
International Society for Behavioral Ecology

Behavioral Ecology (2020), 31(1), 202–212. doi:10.1093/beheco/arz178

Original Article

Effects of condition and sperm competition risk on sperm allocation and storage in neriid flies

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Received 4 April 2019; revised 25 August 2019; editorial decision 13 September 2019; accepted 23 September 2019; Advance Access publication 7 November 2019.

Ejaculate traits can be sexually selected and often exhibit heightened condition-dependence. However, the influence of sperm competition risk in tandem with condition-dependent ejaculate allocation strategies is relatively unstudied. Because ejaculates are costly to produce, high-condition males may be expected to invest more in ejaculates when sperm competition risk is greater. We examined the condition-dependence of ejaculate size by manipulating nutrient concentration in the juvenile (larval) diet of the neriid fly *Telostylinus angusticollis*. Using a fully factorial design we also examined the effects of perceived sperm competition risk (manipulated by allowing males to mate first or second) on the quantity of ejaculate transferred and stored in the three spermathecae of the female reproductive tract. To differentiate male ejaculates, we fed males nontoxic rhodamine fluorophores (which bind to proteins in the body) prior to mating, labeling their sperm red or green. We found that high-condition males initiated mating more quickly and, when mating second, transferred more ejaculate to both of the female's posterior spermathecae. This suggests that males allocate ejaculates strategically, with high-condition males elevating their ejaculate investment only when facing sperm competition. More broadly, our findings suggest that ejaculate allocation strategies can incorporate variation in both condition and perceived risk of sperm competition.

Key words: **condition-dependence, ejaculate, larval diet, rhodamine, sexual selection, sperm competition, *Telostylinus angusticollis***

INTRODUCTION

When a female mates with multiple males, competing ejaculates may temporally and spatially overlap and compete for fertilization of the female's eggs (Parker 1970). Selection, therefore, favors male traits that increase the competitive ability of sperm (Parker 1970; Simmons 2001; Snook 2005; Pizzari and Parker 2009; Manier et al. 2013). Selection may also act on females, and favor the ability to cryptically choose (i.e., bias) fertilization success towards a particular male's sperm (Thornhill 1983; Birkhead and Moller 1993; Eberhard 1996). In many insects, females have multiple long-term sperm storage organs (spermathecae) within their reproductive tracts, where sperm competition is often intensified (Pascini and Martins 2017). Sperm competition has been shown to select for traits that enhance fertilization success when multiple ejaculates co-occur within the female reproductive tract: for example, in lines with experimentally increased rates of polyandry, male and female yellow-dung flies have been shown to evolve larger testes and accessory sex glands, respectively (Hosken et al. 2001). However, how male condition influences ejaculate allocation strategies in response to perceived sperm competition remains poorly understood.

Theory on sperm allocation suggests that males are selected to transfer more sperm when detecting a risk of sperm competition (e.g., as when mating with a previously mated female) than when mating with a virgin female (Parker et al. 1996, 1997; Parker and Begon 1986). This prediction has been supported by empirical work (Gage and Baker 1991; Cook and Gage 1995; Fuller 1998; Aron et al. 2016). However, numerous empirical studies have also failed to support this prediction, bringing the generality of this rule into question (reviewed in Williams et al. 2005). Condition-dependent variation in ejaculate allocation strategies could contribute to variable outcomes of empirical studies. If ejaculate traits are sexually selected and costly, then we should expect these traits to exhibit heightened condition-dependence when compared to most other traits (Andersson 1994; Rowe and Houle 1996; Bonduriansky and Rowe 2005). Theoretical and empirical studies have begun to reveal that both sperm and non-sperm components of the ejaculate can be similarly plastic and condition-dependent in their responses to the developmental

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environment (Wigby et al. 2016). Yet, how variation in male condition shapes ejaculate allocation remains poorly understood.

Condition can be defined as the amount of metabolic resources that an individual has to allocate to all fitness-related traits, which can be affected by nutrient abundance in the environment as well as genes that affect the capacity to extract and metabolize resources (Andersson 1982; Rowe and Houle 1996; Hill 2011). Consistent with predictions, there is evidence that ejaculate properties can vary in response to food availability (Perry and Rowe 2010; Kahrl and Cox 2015; Rahman et al. 2013) and inbreeding depression (Gage et al. 2006; Zajitschek and Brooks 2010; Maximini et al. 2011), both of which likely affect the amount of resources available for a male to invest in reproduction (Mautz et al. 2013). The nature of the relationship between ejaculate quality and investment in other life-history traits is complex (Roff and Fairbairn 2007), and subject to resource allocation trade-offs (Engqvist 2011; Simmons and Emlen 2006). Nonetheless, males that possess more resources (i.e., high-condition males) are still expected to produce larger ejaculates if they experience a lower marginal cost of ejaculate production than do low-condition males (Parker 1990; Tazzyman et al. 2009). This prediction has been supported in diverse taxa, including reptiles (Kahrl and Cox 2015); fish (Rahman et al. 2013) and insects (Watanabe and Hirota 1999; Jia et al. 2000; Ferkau and Fischer 2006; Perry and Rowe 2010; Kaldun and Otti 2016). High-condition males produce bigger ejaculates (Fedina and Lewis 2004; Blanco et al. 2006), transfer larger amounts of sperm (Perez-Staples et al. 2008) and can produce higher quality nuptial gifts (Jia et al. 2000). Most studies manipulate male condition through dietary restriction at the adult stage, so less is known about how the juvenile nutritional environment shapes adult post-copulatory performance, although effects of resource availability during development have been explored in several studies (Amitin and Pitnick 2007; McGraw et al. 2007; Engqvist 2008; Melo et al. 2014; Vega-Trejo et al. 2016; Dávila and Aron 2017). Most of these studies show that poor developmental nutrition has a negative effect on ejaculate size and quality (Delisle and Bouchard 1995; Bissoondath and Wiklund 1996; Amitin and Pitnick 2007). Juvenile nutrition is likely to play an especially important role in shaping the development of adult traits, including ejaculate traits, in holometabolous insects (Macartney et al. 2018).

To understand ejaculate allocation strategies, it is necessary to elucidate how sperm competition risk and condition jointly influence investment in ejaculate traits (Perry et al. 2013). Males in good condition are predicted to invest more in secondary sexual traits (Andersson 1982; Pomiankowski 1987; Cotton et al. 2004). However, selection favors prudent ejaculate allocation strategies (Wedell et al. 2002), so high-condition males might be selected to elevate their ejaculate investment only when perceiving a risk of sperm competition. High-condition males tend to have higher mating rates than smaller males, as seen in antler flies (Bonduriansky and Brassil 2005), European vipers (Madsen et al. 1993), and rhesus macaques (Georgiev et al. 2015). High-condition males might, therefore, be expected to evolve prudent ejaculate allocation strategies that enable them to take advantage of frequent mating opportunities. By contrast, low-condition males might be selected to invest maximally in all matings because their probability of achieving a mating is lower, and they may lack the resources required to elevate ejaculate expenditure even further when facing high sperm competition risk.

Here we assess the effects of male condition and sperm competition risk (i.e., mating sequence) on the amount of ejaculate transferred by males and stored in the female spermathecae in *Telostylinus angusticollis* (Diptera: Neriidae), a species endemic to New South Wales and southern Queensland, Australia. Mating sequence predicts the level of sperm competition, such that males might be selected to invest more ejaculate if they can detect that they are mating second. *Drosophila melanogaster* males have been shown to mark females with pheromones that make the females less attractive to other males and decrease the females' probability of re-mating (Laturney and Billeter 2016). *Telostylinus angusticollis* females are polyandrous, and males could utilize similar chemical cues to detect sperm competition risk. However, male responses to perceived sperm competition risk could depend on male condition. In this species, many aspects of the adult male phenotype and reproductive strategy are strongly influenced by dietary nutrients during development. Increasing the concentration of dietary nutrients during the larval stage results in increased body size and enlarged secondary sexual traits (Sentinella et al. 2013). Nutrient-rich larval diet also enhances sperm motility (Macartney et al. 2018). However, less is known about the effects of larval diet on ejaculate allocation strategies. Males of this species do not impart a nutritious nuptial gift (Bonduriansky and Head 2007), but insect ejaculates contain seminal proteins that could influence female behavior and physiology (e.g., stimulate egg production) and such proteins could be costly for males to produce (Perry and Rowe 2010). The costs of sperm production can also be considerable (Dewsbury 1982; Nakatsuru and Kramer 1982; Van Voorhies 1992; Pitnick and Markow 1994; Pitnick 1996; Olsson et al. 1997; Dowling and Simmons 2012). Yet, males of this species can mate many times within a short time frame (E.L. MacCartney, unpublished data), and this ability might be associated with strategic ejaculate allocation strategies.

Telostylinus angusticollis females possess three spermathecae, two of which are joined by a common spermathecal duct (Figure 1b), and males deposit sperm directly into a spermathecal duct (Bath et al. 2012). Sperm then move in an undulating motion towards the spermathecae (Nicovich et al. 2015) and coalesce on the spermathecal equator (Wylde Z, unpublished data). It is not known whether there is any stratification of competing sperm among these three storage organs, or if the three spermathecae have different functions. For example, some insects (such as lacebugs, Hemiptera:Tingidae) possess "pseudospermathecae" that do not store sperm but instead play a role in the transfer of secretions to eggs traveling down the oviduct (Carayon 1958; Marchini et al. 2010).

We manipulated both early-life condition (by rearing males on a nutrient-rich or nutrient-poor larval diet) and male mating sequence (by mating males 1st or 2nd to a single female) in a fully crossed design. We tracked and quantified competing male ejaculates within each of the three spermathecae using a new method of ejaculate staining with rhodamine dyes, based on previous work by Hayashi and Kamimura (2002). Differences in ejaculate storage patterns between the different spermathecae might signify a difference in spermathecal function or male ejaculate transfer strategy. We predicted that males possessing more resources (i.e., high-condition males) would transfer larger ejaculates and asked whether condition-dependent ejaculate allocation patterns are also influenced by mating sequence.

MATERIAL AND METHODS

Experimental animals

The flies used in these experiments were of the third and fourth generations of laboratory-reared stock populations that originate from Fred Hollows Reserve, Randwick, NSW, Australia

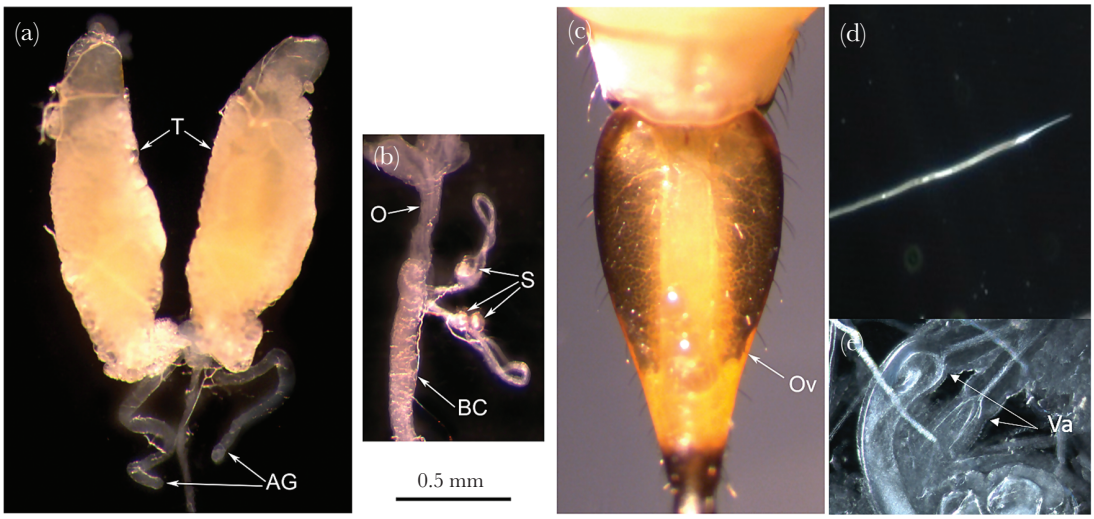

Figure 1
Reproductive organs of *T. angusticollis*. (a) Male testes and accessory glands (AG). (b) Female bursa copulatrix (BC), spermathecae (S) and oviduct (O). (c) Female oviscape. (d) Mature spermatozoon (acrosome and midpiece shown). (e) spermathecal duct valves (Va) and junction to bursa. The Image in panel b is modified from Bath et al. 2012).

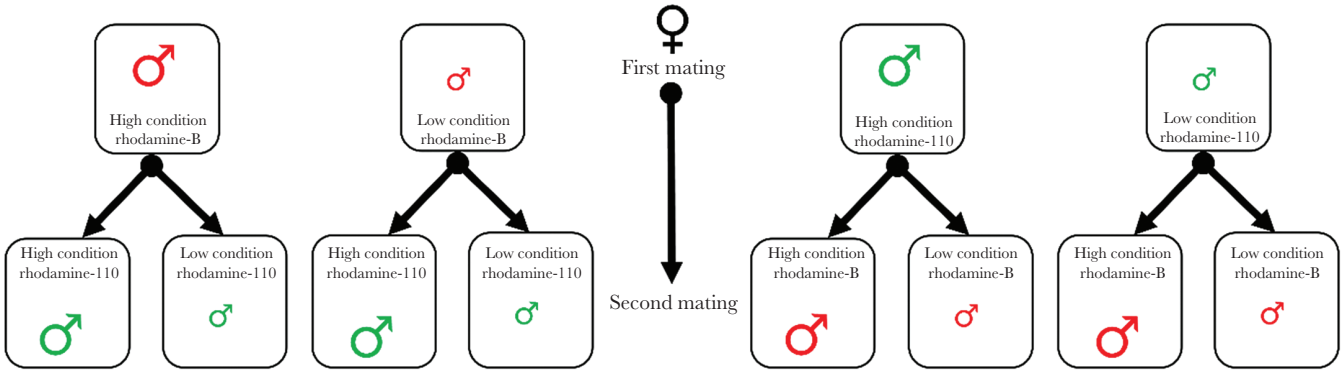

Figure 2
Experimental design. Fully factorial design for mating sequence, larval diet (assumed to influence condition), and rhodamine dye (red or green) treatment (high condition = large male symbols; low condition = small male symbols).

(33°54′44.04″S 151°14′52.14″E). To avoid inbreeding depression, flies in this stock were periodically replenished with wild-caught individuals from the same source population. All larvae were reared in climate chambers at 25 ± 2 °C with a 12:12 photoperiod and moistened with water every 2 days. The first block of this experiment was completed in March 2016 on the third-generation cohort and the second block was completed in May 2017 using the fourth-generation cohort.

Larval diet manipulation

We employed a 2 × 2 factorial design with manipulation of male larval diet (as a means of generating males of varying condition and body size) crossed with a manipulation of mating sequence (Figure 2). We manipulated the quantity of resources available to larvae during development by rearing flies on either a nutrient-rich or nutrient-poor diet based on Sentinella et al. (2013). All diets consisted of a base of 170 g of coco peat moistened with 600 mL of reverse osmosis-purified (RO) water. The "rich" larval diet consisted of 32.8 g of protein (Nature's Way soy protein isolate; Pharm-a-Care, Warriewood, Australia) and 89 g of brown sugar (Woolworths Essentials Bonsucro brand), the "standard" larval diet consisted of 10.9 g of protein and 29.7 g sugar, and the "poor" larval diet consisted of 5.5 g of protein and 14.8 g sugar. These nutrients were mixed into the cocopeat and water using a handheld blender and frozen at −20 °C until the day of use. All females used in the experiments described below were reared on a "rich" larval diet. Upon adult emergence, flies were separated by sex and diet treatment and kept in populations of 30 ± 3 individuals per 8L cage with sugar/yeast and water (ad libitum) for 15 days. Females were additionally provided oviposition medium to hasten vitellogenesis.

Labeling ejaculates with rhodamine

After 15 days, focal males were transferred into individual 120 mL containers fitted with 2 × 2 mL Eppendorf tubes of RO water (gravity-fed from container lid) with a cocopeat substrate (to retain moisture) and provided with the high-nutrient medium (described above) to which either rhodamine 110 (RH_{110}) (green) or rhodamine B (RH_B) (red) had been added. The males were left to feed on these media for 5 days prior to being paired with a female. Most fluorescent dyes are toxic and are applied postmortem, whereas rhodamine dyes appear to have little toxicity. A study comparing the toxicity of multiple rhodamine dyes in the house fly *Musca domestica*, showed no significant differences in mortality rates when flies were orally fed the dyes for up to 5 days (Respicio and Heitz

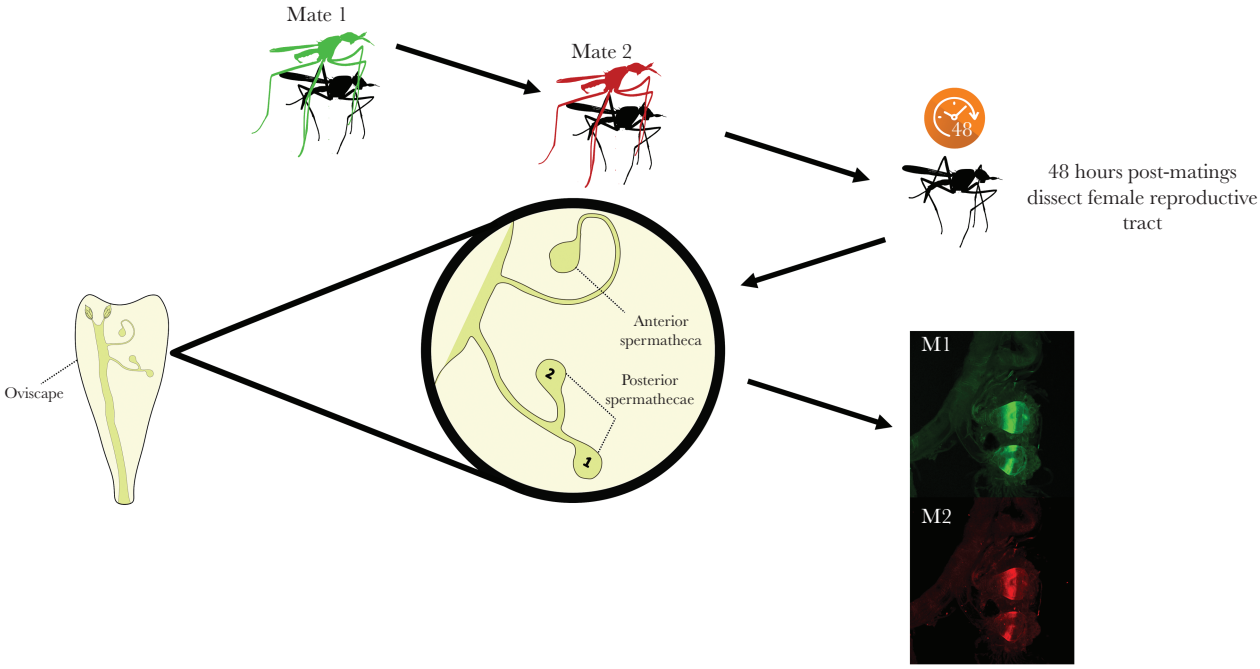

Figure 3
Experimental workflow. Two males reared on rich or poor larval diet and fed red or green rhodamine dye were mated sequentially to a single female in a fully factorial design, as shown in Figure 2. The female reproductive tract was dissected 48 h after the second mating. Finally, spermathecae were imaged sequentially for each dye treatment using confocal microscopy to obtain fluorescent signal of competing male ejaculates. M1 and M2 indicate an example of ejaculate signal in the posterior spermathecae where the first mate (M1) was fed RH110, and the second (M2) to mate was fed RHB. The image sequence was randomized for dye treatment filter to minimize any effects of photobleaching that can occur from confocal microscopy.

1981). These dyes have an affinity to proteinaceous compounds and form stable covalent bonds and so make it possible to label ejaculates and track competing sperm in the female reproductive tract without the need to genetically modify organisms to produce fluorescent probes such as Green or Red Fluorescent Protein (GFP/RFP) (Manier et al. 2010). Artificial injection of RH_B into the genital tract of a female leafhopper (*Bothrogonia ferruginea*) resulted in this dye being incorporated into ovarioles (Hayashi and Kamimura 2002). In tobacco moths (*Heliothis virescens*), males fed rhodamine B transferred spermatophores that emitted a fluorescent signal under UV light, with no reduction in lifespan (Blanco et al. 2006). The rhodamine dyes bind to all proteinaceous components of the ejaculate, so fluorescent signal intensity is proportional to the total amount of ejaculate transferred including both seminal fluid and sperm. RH_B and RH_{110} were also chosen because of their structural similarity (almost identical apart from their attached fluorophore) to minimize any biochemical differences in their uptake/ attachment to ejaculate proteins. We observed no obvious effects of RH_B or RH_{110} on behavior or mortality in *T. angusticollis* (Z. Wylde, personal observations).

Mating sequence manipulation

We randomly assigned adult males from each larval diet treatment to a mating sequence position of either P1 or P2 (first or second to mate). Two males (previously fed different rhodamine dyes) were mated to a single rich diet female in quick succession in a fully factorial design incorporating all combinations of male condition and mating sequence (Figure 3). This enabled us to examine the main effects and interaction of mating sequence and larval diet treatments. To control for any effects of red versus green rhodamine dye, we set up approximately equal numbers of treatment and pairings with $P1_{red}/P2_{green}$ and $P1_{green}/P2_{red}$ dye combinations. We also control for dye statistically (see below).

Males were allowed up to 10 min to achieve copulation with the focal female within a scintillation vial in the presence of a food source (which stimulates sexual activity), and latency to mating and copulation duration were recorded. Latency to mate was timed from when individuals were placed within the scintillation vials (and allowed 10 s to adjust) until the initiation of copulation was observed. The beginning of copulation was defined when the male was observed to mount the female and lock his epandrium into position onto the female oviscape, and the end of copulation was defined as the point when the male withdrew his genitalia from the female oviscape.

Mean copulation duration of *T. angusticollis* males is approximately 75 s (Bath et al. 2012), so all pairs that separated before 20 s were discarded. After the first mating was completed, the first male was immediately removed from the vial and frozen, and the second male was introduced. Following the second mating, the second male was removed and frozen and females were immediately placed in individual 120 mL containers with a substrate of moistened cocopeat and a Petri dish containing a sugar/yeast mixture, and 2 × 2 mL Eppendorf tubes of RO water, but without oviposition medium. *Telostylinus angusticollis* females do not oviposit unless oviposition medium is provided, and we wanted to prevent females from immediately using any of the sperm received from their two mates in order to quantify patterns of sperm storage in the spermathecae. The mated females were kept in these containers for 48 h to allow for the ejaculates to reach the spermathecae. Thorax length was measured as an index for body size for all focal males and the females they were paired with.

Sample preparation

After 48 h females were anesthetized (but not killed) by placing them for 5 min into a −20 °C freezer and their reproductive tracts were then carefully dissected in saline solution and mounted in Prolong Diamond Antifade mountant (to reduce photobleaching) on 1–1.2 mm glass slides with 22 × 22 mm coverslips (Labserv). All mounted samples were allowed 24 h to cure at room temperature (in absence of light), stored at −80 °C, and imaged within a week of preparation.

Quantifying amount of stored ejaculate

To quantify the relative amount of ejaculate from each competing male within the spermathecae, fluorescence signal intensities for each dye treatment were obtained using a Leica TCS SP5 WLL STED confocal microscope with Hybrid (HyD) detectors for enhanced sensitivity. Males that were fed RH_B had ejaculates with an excitation of 540 nm (red spectrum), whereas males fed RH_{110} had ejaculates that showed excitation at 488 nm (green spectrum). The emission of photons for both dye treatments were counted using Hybrid detectors with spectral ranges of 560–630 nm for RH_B and 500–550 nm for RH_{110} (fluorophores excited at 540/488 nm emit photons in these ranges). All images were taken at 40× with a 0.7 HCX Plan Fluotar AIR 0.4 mm objective lens. For all images, the White Light Laser (WLL) gain was set to 30% and laser strength to 40% with a laser speed 80 Hz. All images were taken in 12-bit 514 × 514 resolution. Sequential scans (between frames) were used for each dye treatment filter (to minimize any bleed-through) with a line accumulation of 14, frame average of 4 and a scanning speed of 100 Hz to maximize signal. A maximum of 4 images were taken for each sample and randomized in their acquisition sequence for the RH_{110} and RH_B filter sets to minimize any effect of photobleaching.

All images were captured using Leica Application Suite *Advanced fluorescence* (version 2.2.3.9723; Leica Microsystems, 1997–2013). All images were subsequently analyzed using ImageJ 1.51d (Schindelin et al. 2012). Signal intensity measures (integrated density) were captured by a single hand selection by tracing the perimeter of each individual spermatheca to create a region of interest (ROI) for each focal female. The same ROI was used for both RH_B and RH_{110} images, which were imaged in the exact same positions. This ensured consistent pixel samples within each focal sample to avoid variability that might arise from multiple hand selections of the same spermathecae. Repeatability of ejaculate signal for multiple hand traces of a single spermatheaca was 0.99 (n = 20). Many insect tissues including muscles, unsclerotized body parts (Friedrich et al. 2014) and the cephaloskeleton (Grzywacz et al. 2014) will fluoresce under different wavelengths of light without staining. Although auto-fluorescence of tissue was negligible, we controlled for any background noise by taking five measurements of spermathecae from six virgin females using the same settings for each dye treatment as above. The average of the integrated density measures from these images was then deducted from each ejaculate measure according to dye treatment as a way to correct for any differences in auto-fluorescence that might occur for the different dye settings.

Statistical analyses

All missing values for thorax length (males that escaped after mating; Rich-diet n = 6, Poor-diet n = 8) were replaced with mean values for the relevant larval diet treatment (Supplementary Figure S1). Fluorescence intensity data were used as a proxy for ejaculate amount. Replicates with outlier values (i.e., values > 2 standard deviations from the mean) were removed, and intensity data were then standardized (i.e., converted to z-scores) within dye treatment (red or green) × block combinations (first or second) to eliminate any mean differences in intensity between samples labeled with RH_B or RH_{110}.

We fit models with fixed effects of larval diet (LD), mating sequence (MS) and their interaction, thorax length (centered within larval diets, CMTL) to account for variation in male body size within larval diet treatments, copulation duration (CD) to determine whether increased ejaculate expenditure was a function of increased mating time or sperm transfer rate, and block (BL) to account for any variability that might occur between experimental blocks. Male thorax length values were mean-centered within larval diet treatments to eliminate collinearity with the categorical effect of larval diet. Body size is often correlated with female fecundity, and latency to mate (LT) could affect ejaculate transfer, so both female body size (FTL) and LT were initially included as covariates in our models. These covariates were not found to have any significant effects and were subsequently dropped from further analyses. Mating pair ID and pairing date were included as random effects in all models. In addition, because the effects of larval diet could be mediated at least in part through body size, we also modeled our data using uncentred thorax length as a predictor instead of larval diet; these analyses yielded qualitatively similar results (Supplementary Material).

All statistical analyses were performed using R 3.3.2 (R Core Team 2012) and the R package "lme4" (Bates et al. 2015) was used to fit linear mixed models (LMM). All fixed effects in our linear mixed models were tested using the "lmerTest" package (Kuznetsova et al. 2017), with type III ANOVA F statistics based on Satterthwaite approximations.

RESULTS

Body size

We found that male larval diet affected adult body size: males reared on a nutrient-poor larval diet had a smaller thorax length than did males reared on a nutrient-rich larval diet (Anova, $F(1, 190) = 281.5$, $P \leq 0.0001$; Figure 4).

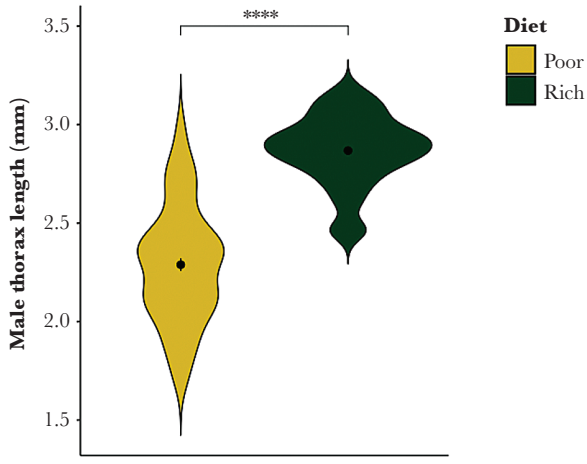

Figure 4
The effects of larval diet quality on male thorax length. The violin plot outlines illustrate the kernel probability density, that is, the width of the outlined area represents the proportion of the data located there.

Copulatory behaviors

Male larval diet affected males' latency to mate. Males reared on a poor larval diet took longer to start mating. There was no effect of male larval diet on copulation duration. There were no effects of mating sequence, nor the interaction of larval diet and mating sequence, for either latency to mate or copulation duration. Likewise, male body size within larval diet treatments had no effect on latency or copulation duration (Table 1, Figure 5).

Ejaculate within the spermathecae

We tested effects of larval diet, mating sequence, and their interaction on the amount of ejaculate present within each spermatheca (quantified as fluorescence signal intensity from the rhodamine dyes). The larval diet and mating sequence interaction was significant for both posterior spermathecae, reflecting a relative increase in the amount of stored ejaculate from high-condition males when mating second (Table 2; Figure 6). This interaction was non-significant for the anterior spermatheca (Figure 6c) but the pattern appeared qualitatively similar to that for the posterior spermathecae (Figure 6a). The interaction between mating sequence and male larval diet persisted when copulation duration was added to the model, and became significant for the second posterior spermatheca, indicating that the effects are not mediated entirely by copulation duration. Copulation duration had a significant effect on the amount of ejaculate in both posterior spermathecae but not the anterior spermatheca (Table 2). Additionally, the total amount of ejaculate did not differ significantly among the three spermathecae (Anova: $F_{2,\ 573} = 2.331$, $P = 0.098$; Figure 7).

Table 1

Linear mixed model results for latency to mate and copulation duration (including larval diet)

	Latency to mate			Copulation duration		
Fixed effects	Estimate	SE	df	Estimate	SE	df
(Intercept)	**178.08**	**29.79*****	36.45	**60.259**	**3.781*****	49.26
Male larval diet	**−87.87**	**25.18*****	176.63	−1.542	3.598	177.75
Mating sequence	−30.66	25.89	150.29	1.949	3.668	148.53
Male thorax length (centered within larval diets)	−43.80	39.51	183.62	−2.971	5.589	183.06
Block	28.65	31.22	17.71	**−6.786**	**3.720.**	20.11
Mating sequence: Male larval diet	42.04	35.85	179.05	0.403	−0.047	180.91

Effects with $P < 0.05$ are in bold. Significance codes: 0.0001 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1.

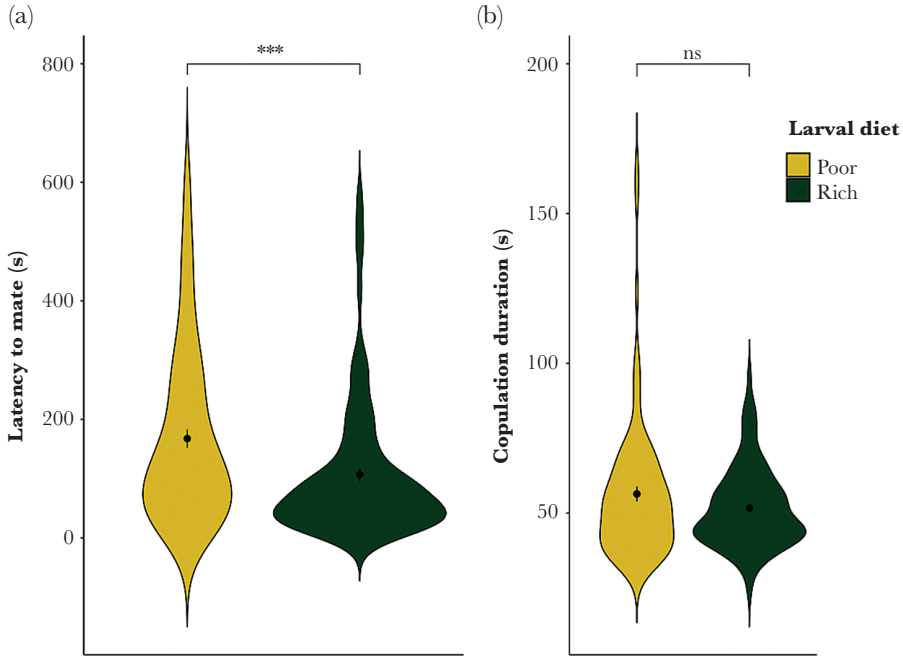

Figure 5

(a) Latency to mate in males reared on rich and poor larval diets. (b) Copulation duration in males reared on rich or poor larval diets. Bars show mean ± SEM. The violin plot outlines illustrate the kernel probability density (i.e., the width of the outlined area represents the proportion of the data located there). Bracket and asterix above indicate statistical difference as indicated in Table 1.

Table 2

Linear mixed models for effects of male thorax length (centered), mating sequence and copulation duration on ejaculate storage

	Posterior spermatheca 1			Posterior spermatheca 2			Anterior Spermatheca		
Fixed effects	Estimate	SE	df	Estimate	SE	df	Estimate	SE	df
(Intercept)	−0.293	0.338	54.55	−0.166	0.344	42.51	0.063	0.351	44.02
Male larval diet	−0.154	0.138	115.52	−0.089	0.123	101.56	−0.191	0.129	105.66
Mating sequence	−0.162	0.131	106.33	**−0.220**	**0.117.**	93.56	−0.123	0.125	96.78
Copulation duration	**0.008**	**0.003***	132.53	**0.071**	**0.003***	113.63	0.003	0.003	121.35
Male thorax length (centered)	0.071	0.230	127.36	−0.163	0.208	110.60	0.119	0.220	117.12
Block	−0.077	0.333	20.41	−0.137	0.364	20.07	−0.192	0.366	19.79
Mating sequence: Male larval diet	**0.463**	**0.199***	120.98	**0.376**	**0.178***	103.21	0.369	0.188·	108.49

Bold values are significance codes: 0.0001 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1.

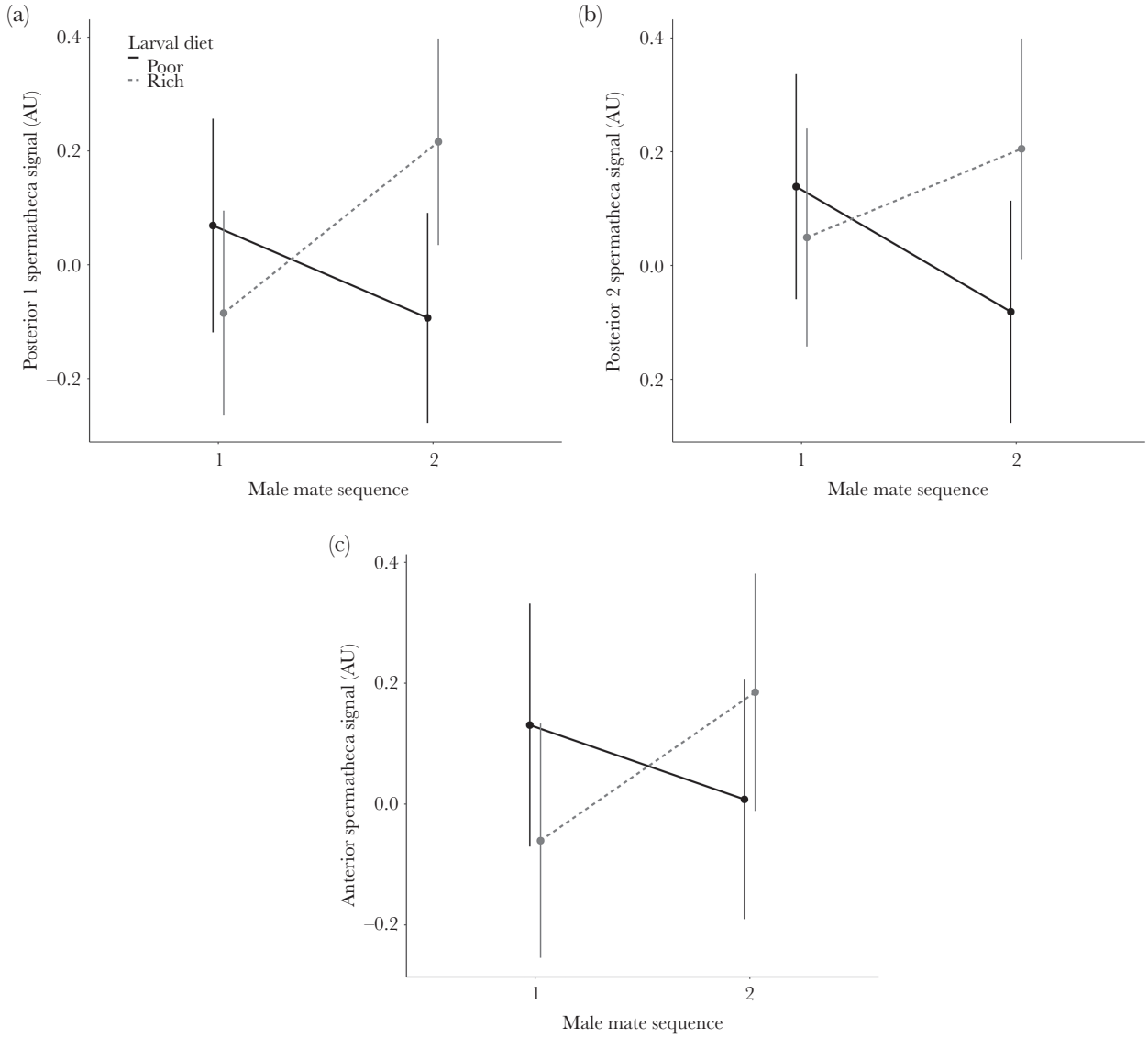

Figure 6

Interaction between male larval diet and mating sequence on the amount of ejaculate within each spermatheca. (a) ejaculate within the posterior "1" spermathecal (doublet). (b) Ejaculate within the posterior "2" (doublet). (c) ejaculate within the anterior spermathecal (singlet). Lines represent least squares mean ejaculate amounts measured in arbitrary units (fluorescence) ± SEM. Solid black lines represent the ejaculate from poor-diet males (low condition) and dashed gray represent rich-diet male ejaculate (high condition). Male mate sequence is characterized as either 1 (first to mate) or 2 (second to mate). These results show that rich-diet males generally transferred more ejaculate. These patterns also provide some evidence that, when mating second, rich-diet males increased their ejaculate investment more than poor diet males did.

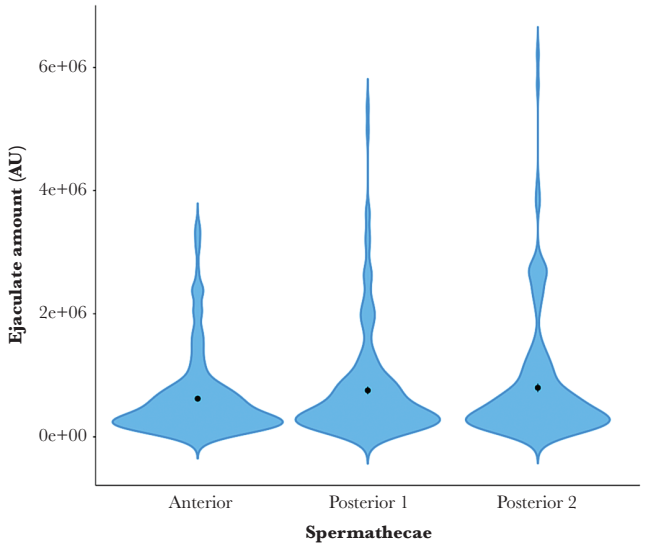

Figure 7
Total amount of ejaculate (arbitrary fluorescence signal units, AU) within each of the three spermathecae. The violin plot outlines illustrate the kernel probability density (i.e., the width of the outlined area represents the proportion of the data located there). Points and bars within the violin plots indicate mean + SEM.

DISCUSSION

Our results provide evidence that male ejaculate allocation strategies in response to perceived risk of sperm competition are condition-dependent. For males reared on a rich larval diet, the amount of ejaculate stored in both of the posterior spermathecae was greater when mating second (i.e., under risk of sperm competition from the previous male) than when mating first (i.e., with a virgin female). By contrast, for males reared on a poor larval diet, the effect of mating sequence on the amount of ejaculate transferred to the posterior spermathecae was weaker, as indicated by a larval diet by mating sequence interaction. A qualitatively similar pattern was observed for the anterior spermatheca, although the interaction was nonsignificant. Males reared on a rich larval diet were also able to initiate mating more quickly, perhaps because such males were more vigorous or more attractive to females.

One way that males could change allocation strategies is by increasing copulation duration. Copulation duration was found to significantly relate to the amount of ejaculate, where the longer a male copulated with a female, the more ejaculate was stored in the two posterior spermathecae. Copulation duration had no effect on the amount of ejaculate within the anterior spermatheca (most distal to the bursa copulatrix), suggesting that another ejaculate trait, perhaps sperm motility, or a female-mediated trait might also affect ejaculate storage in the anterior spermathecae. However, with copulation duration in the model, the interaction between mating sequence and larval diet was significant for both posterior spermathecae, suggesting that treatment effects on ejaculate size were not entirely mediated by differences in copulation duration but resulted from an increase in the rate of ejaculate transfer (seminal fluid, sperm, or both). This suggests that high-condition males allocate ejaculates strategically, elevating their rate of transfer only when perceiving a risk of sperm competition. When mating with virgin females (and thus perceiving low risk of sperm competition), this strategy might enable high-condition males to save up ejaculate resources for future matings. If high-condition males experience a high mating rate in the wild, such males might benefit by adjusting ejaculate allocation to each mating based on the perceived risk of sperm competition in order to save up ejaculate resources for subsequent matings. Conversely, if low-condition males experience a very low mating rate in the wild, such males may be selected to invest maximally in each mating. Low-condition males may have low mating rates in the wild because they perform poorly in combat and territory defense (Hooper et al. 2017), and because neriid females appear to discriminate against such males (Fricke et al. 2015).

The observed interaction of larval diet and mating sequence could be driven by a dosage mechanism (whereby high-condition males deposit larger ejaculates when mating second) and/or by condition-dependent variation in sperm quality (whereby low-condition males defense sperm that is less likely to reach the spermathecae). High-condition *T. angusticollis* males transfer sperm with a higher tail-beat frequency (Macartney et al. 2018). Low-condition males may, therefore, transfer less competitive sperm that are less likely to enter the spermathecae when compared to sperm of high-condition males. Aberrant sperm (caused by errors in spermatogenesis) with abnormal flagella and, therefore, lower motility have been observed in many insect species (see review Dallai et al. 2016). Similar results have been obtained in fish, where low-condition males produce less motile sperm (Locatello et al. 2006; Burness et al. 2008; Rahman et al. 2013; Macartney et al. 2018). Whether variation in condition resulting from our larval diet manipulation can also influence the quality and production of seminal fluid remains to be explored in *T. angusticollis*. Because our imaging technique was unable to differentiate between sperm and semen, we were not able to examine how the ejaculate composition may have changed in response to mating sequence.

Sperm storage patterns in the spermathecae could affect competitive fertilization success. The insect spermatheca is thought to be involved in sperm maturation or activation and long-term storage (Klowden 2006). Once eggs have matured and been released, sperm swim back through the spermathecal ducts to the site of fertilization, which is typically thought to be the oviduct at the junction of these ducts (Pascini and Martins 2017). We found that ejaculate amounts deposited into all three spermathecae were very similar (Figure 7), but our results also provide tentative evidence of differential sperm storage patterns across these spermathecae. The functional consequences (if any) of such variation in sperm storage across spermathecae for cryptic female mate choice and male competitive fertilization success remain to be determined. It is not clear whether males can influence how their ejaculates are distributed among the three spermathecae, but it is possible that males might benefit by depositing equal amounts of ejaculate into all spermathecae to decrease variance in reproductive success when a female releases sperm from different spermathecae. Our behavioral assay design represents a very simplified mating environment with little opportunity for mate choice and a structurally simple arena with no refuges for females to escape male coercion, thereby limiting any effects that pre-copulatory processes may have on ejaculate storage.

Our findings also suggest that males are able to assess a female's mating history and differentiate between virgin and mated females. Cuticular hydrocarbons (CHCs) could be used by males to assess female mating history. In *D. melanogaster*, males transfer anti-aphrodisiac pheromones (CHCs) to the female reproductive tract and cuticle, and these CHCs reduce female attractiveness to other males (Laturney and Billeter 2016). It is possible that neriid flies possess a similar mechanism that allows a male to assess a female's

reproductive status and change his ejaculate allocation strategy accordingly.

Our results suggest that the condition-dependent ejaculate budget of a *T. angusticollis* male affects his ability to respond to sperm competition. Non-sperm seminal fluid components appear to be especially costly and limiting in insects (Marcotte et al. 2007; Reinhardt et al. 2015), and the costs and benefits of varying ejaculate compositions have been posited to select for plasticity in the structure of ejaculates (Perry et al. 2013). Such plasticity is consistent with theory (Parker 1990), as suggested by previous empirical findings, particularly in insects that produce nuptial gifts (spermatophores) (Jia et al. 2000; Blanco et al. 2009; Perry and Rowe 2010). Perhaps low-condition males transferred more sperm at the expense of non-sperm components. Non-sperm components are known to make up the bulk of ejaculate composition in many species, while increased sperm numbers have been shown to contribute very little to ejaculate mass (Perry and Rowe 2010). More costly ejaculate components are expected to be more strongly condition-dependent (Andersson 1982; Rowe and Houle 1996), but very little is known about the actual costs of seminal proteins and if their production is energetically more expensive than that of sperm.

Previous studies have shown status-dependent sperm investment (fowl; *Gallus gallus*; Pizzari et al. 2003), female influence on sperm storage and preferences for larger male sperm (dung fly; *Scathophaga stercoraria*; Ward 1993), and the condition-dependence (investigated using adult diet manipulation) of ejaculate size and composition (ladybird beetle, *Adalia bipunctata*; Perry and Rowe 2010). We present the first evidence that males adjust their ejaculate allocation strategy in response to perceived sperm competition in a condition-dependent manner. Our study is also one of the first to show that larval (as opposed to adult) nutrition can affect adult sperm allocation patterns.

We used a recently developed method that utilizes rhodamine fluorophores (incorporated into adult diet) for the labeling of ejaculates. Rhodamine fluorophores appear to be a promising method for staining ejaculates because of their affinity for proteinaceous compounds, and most importantly, their non-toxicity which allows for a relatively affordable and simple labeling of insect ejaculates. This method offers an alternative to genetically altering sperm to express green fluorescent protein/ red fluorescent sperm heads (GFP/RFP) (Manier et al. 2010). The rhodamine labeling technique labels both sperm and seminal proteins. Our analysis assumes that the dyes bind with equal affinity to all ejaculates and do not interact with female tissues. The chemical structures of RH_B and RH_{110}, however, are very similar (Beija et al. 2009), reducing the possibility of differences in fluorescence as a result of discrepancies in dye attachment.

CONCLUSIONS

Revealing how condition-dependent traits such as ejaculate amount change with social context is important in better understanding responses to sperm competition. We provide evidence that ejaculate allocation is complex and is affected by an interaction of sperm competition risk and condition. Further studies examining how male condition and social environment interact to affect ejaculate allocation and storage will contribute to understanding the mechanisms that generate differences in paternity amongst competing males.

SUPPLEMENTARY DATA

Supplementary data are available at *Behavioral Ecology* online.

FUNDING

This work was supported by the Australian Research Council grant awarded to R.B. (grant number DP170102449).

The authors would like to extend their thanks to the other members of the Bonduriansky lab for all support and valuable discussions during this experiment. Special thanks to the BMIF facility at UNSW for their helpful advice and training in confocal microscopy.

Conflict of Interest: The authors declare that there was no conflict of interest regarding the publication of this article.

Data Accessibility: Analyses reported in this article can be reproduced using the data provided by Wylde et al. (2019).

Handling editor: Luke Holman

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