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Analysis of correlated responses in key ejaculatory traits to artificial selection on a diversifying secondary sexual trait

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ABSTRACT

Positive genetic covariance between male sexual display traits and fertilizing capacity can arise through different mechanisms and has important implications for sexual trait evolution. Evidence for such genetic covariance is rare, and when it has been found, specific physiological traits underlying variation in fertilization success linked to trait expression have not been identified. A previous study of correlated responses to bidirectional artificial selection on the male sex comb, a secondary sexual trait, in *Drosophila bipectinata* Duda documented a positive genetic correlation between sexual trait size and competitive fertilization success, and found that transcript levels of multiple seminal fluid proteins (SFPs) were significantly increased in the large sex comb (high) genetic lines. These results suggest that changes in SFP activity may be a causal factor underlying the increased fertilizing capacity of high line males. Here, we tested for correlated responses to this selection in a suite of additional reproductive traits, measured in the context of variation in male age and exposure to rivals. Whereas several traits including sperm length, number and viability, and accessory gland size, increased with age, only sperm viability was influenced by selection treatment, but in complex fashion. Sperm viability of high line males surpassed that of their smaller-combed counterparts when they had been housed with rivals and were 5–6 days old or older. Interestingly, this interaction effect was evident for sperm sampled from the female seminal receptacle, but not from the male seminal vesicles (where sperm have yet to be combined with accessory gland products), consistent with the differential SFP activity between the lines previously found. Our results suggest that differences in sperm quality (as viability) may be a contributing factor to the positive genetic correlation between sexual trait size and competitive fertilization capacity in *D. bipectinata*.

1. Introduction

Polyandry, the occurrence of multiple matings by females during a single reproductive cycle, is widespread among animals (Kvarnemo and Simmons, 2013; Simmons, 2005). When polyandry results in the overlap of ejaculates of different males within the female reproductive tract, it sets the stage for sexual selection to operate during and after copulation (Andersson, 1994; Simmons, 2001; Thornhill and Alcock, 1983). This selection, called postcopulatory sexual selection, comprises sperm competition, where the sperm of different males vie for fertilization opportunities (Parker, 1970), and “cryptic” female choice, where females bias sperm use in favor of certain males over others (Thornhill, 1983). These mechanisms are responsible for a variety of physiological, morphological and behavioral adaptations closely tied to insemination and fertilization in both males and females (Birkhead and Kappeler, 2004; Birkhead and Møller, 1998; Eberhard, 1985; Eberhard, 1996; Sirot et al., 2007; Snook, 2005; Swanson and Vacquier, 2002; Wolfner, 2002). A topic of intensifying interest is the relationship of postcopulatory sexual selection to male secondary sexual traits (Birkhead and Pizzari, 2002; Evans and Garcia-Gonzalez, 2016; Hosken et al., 2008; Lüpold et al., 2014; Simmons et al., 2017), that class of traits traditionally ascribed to precopulatory sexual selection arising from female mate selection and male-male competition (Andersson, 1994; Darwin, 1871).

On the one hand, we may expect the relationship between pre- and postcopulatory adaptations to be governed by resource allocation trade-offs and to lead to negative covariation between them (Evans and Garcia-Gonzalez, 2016; Pomiankowski and Wedell, 2021; Simmons et al., 2017). If the development and maintenance of different fitness-related traits draw from a common pool of limiting resources within the organism, trade-offs in theory could arise between them, imposing a constraint on their expression and evolution (Reznick, 1992; Zera and Harshman, 2001). Negative relationships between costly secondary

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sexual traits and ejaculatory traits have been documented in a variety of taxa (Evans and Garcia-Gonzalez, 2016; Lüpold et al., 2014; Simmons et al., 2017).

Negative correlations between pre- and postcopulatory traits are not always found (Mautz et al., 2013), however, suggesting that the expression of trade-offs between these traits is not universal, or that trade-offs are masked by other factors (Simmons et al., 2017; van Noordwijk and de Jong, 1986). For example, when the development of specific pre- and postcopulatory traits do not coincide, occurring within different time frames of the life cycle, and even if both traits require non-trivial resource allocation (Macartney et al., 2019), direct resource competition between them may not occur (Nijhout and Emlen, 1998; Zera and Harshman, 2001). Thus, the relationship between pre- and postcopulatory traits is not always expected to be negative, and in fact, a number of distinct evolutionary mechanisms predict positive relationships to develop between them (see below).

Positive covariation between pre- and postcopulatory traits, although clearly not expected to be ubiquitous, does occur in a variety of taxa (Mautz et al., 2013; Simmons et al., 2017), and its existence has important implications for the evolution of sexual traits and related phenomena. For example, when male ornamentation and ejaculate quality scale positively in the population, females mating with the most ornamented males may be rewarded with fertility assurance (Møller and Jennions, 2001; Sheldon, 1994). Positive phenotypic correlations between pre- and postcopulatory traits have been documented in a variety of vertebrate and invertebrate species (Cotton et al., 2010; Evans et al., 2003; Forstmeier et al., 2017; Malo et al., 2005; Peters et al., 2004; Pitcher et al., 2007; Reaney and Knell, 2015).

Evidence for positive *genetic* correlations is scant. In the hubbara bustard, *Chlamydotis undulata undulata*, courtship display is genetically correlated with the number of sperm per ejaculate (Chargé et al., 2013). In crickets, *Gryllus bimaculatus*, male body size and aggressiveness have been found to be positively genetically correlated with sperm number (Tuni et al., 2018). In *Drosophila simulans*, family-level (genotypic) covariance between male mating speed (a surrogate of male attractiveness) and competitive fertilization success was found, suggesting the presence of a genetic correlation between these traits, although the precise male traits conferring attractiveness or sperm competitiveness were not identified (Hosken et al., 2008). In *D. bipectinata*, male sex comb size (a morphological secondary sexual trait) has been shown to be positively correlated with competitive fertilization success also across families, and as in the *D. simulans* study, the physiological basis of the enhanced fertilization success was not elucidated (Polak and Simmons, 2009). Finally, across species of Old World leaf warblers, positive correlations have been found between song parameters and sperm length and testis size, suggesting that such patterns of covariation may also be occurring among genotypes within species (Supriya et al., 2018). However, intraspecific correlations were not estimated in this study, which is an important consideration in the present context since interspecific correlations may not be assumed to reflect intraspecific patterns. In any event, the between-species correlations were weak, exhibiting border-line statistical significance or nonsignificance (Supriya et al., 2018).

Positive genetic covariance between pre- and postcopulatory traits may be expected to arise from different mechanisms. One common postulate is that a mutual dependency of these traits on body condition or nutritional state may drive this covariance (Bakker and Pomiankowski, 1995; Mautz et al., 2013; Tuni et al., 2018). The argument is that if there is high variation in resource acquisition ability among genotypes in the population, then the resulting variation in condition itself drives the establishment of positive genetic covariation between pre- and postcopulatory traits (Tuni et al. 2018). Alternatively, positive genetic covariation could arise through the build-up of linkage disequilibrium between alleles influencing pre- and postcopulatory traits as a result of positive epistasis (Polak et al., 2021). Such build-up may develop over time when the most sexually adorned (attractive) males on average face an intensified sperm competitive environment (Polak et al. 2021). It has also been suggested the traits may come to be genetically "integrated" (*sensu* (Cheverud, 1996)) through correlational selection (Birkhead and Pizzari, 2002).

Potential mechanisms aside, the existence of a genetic correlation is important to consider in its own right because when present, indirect postcopulatory selection could then promote shifts in secondary sexual trait expression. Although theoretically plausible, the hypothesis that postcopulatory sexual selection might amplify evolutionary pressure on elements of ornamental traits is largely untested (Andersson and Simmons, 2006; Birkhead and Pizzari, 2002). More studies that test for genetic coupling between male ornamentation and postcopulatory physiological traits are needed.

Recently, a genetic correlation was revealed between ornament expression and competitive fertilization success in *Drosophila bipectinata* Duda (Diptera: Drosophilidae) (Polak et al., 2021). In this study, bidirectional artificial selection was applied on a field-fresh population for 11 consecutive generations on body-size specific sex comb size, successfully generating strongly divergent replicate large-combed (high) lines and small-combed (low) lines. In subsequent fertilization assays using doubly mated females, high-line male exhibited superior competitive fertilization success relative to low-line males, suggesting a positive genetic correlation between sex comb size and competitive fertilization success, corroborating previous work showing positive family-level covariation between these traits (Polak and Simmons, 2009). Transcriptional (RNAseq) characterization of the replicate divergent lines revealed that seminal fluid proteins (SFPs) were consistently upregulated in the high lines relative to the low lines, identifying a potential mechanism for the superior fertilizing capacity of the high line males. This possibility was supported by the finding that large-combed lines maintained their superior fertilizing capacity despite surgical reduction of sex comb size, weakening the alternative hypothesis that the enlarged sex comb *per se* was responsible for the enhanced fertilization success (e.g., (Eberhard, 1996)), for example, via enhanced tactile stimulation of the female with the combs during pre-copulatory courtship (Hurtado-Gonzales et al., 2014).

Here, we utilize the same *D. bipectinata* selection lines generated by Polak et al. (2021) to investigate a suite of male physiological traits that may have contributed to the increased competitive fertilization success of the large-combed (high) lines relative to the small-combed (low) lines. In performing these assays, we aimed to acquire a more complete understanding of the link between secondary sexual trait expression and male fertilizing capacity. The sex comb in *D. bipectinata* is a secondary sexual trait, comprised of modified bristles or "teeth" on the forelegs of males (the sex comb is absent in females). Within the genus *Drosophila*, the sex combs occur in the *melanogaster* and *obscura* species groups, and exhibit remarkable interspecific diversification in shape and size among even closely related taxa (Bock 1971; Bock and Wheeler 1972; Kopp and True, 2002), paralleling that seen in ornamental traits of animals generally (Andersson, 1994). The sex combs are used in courtship in very different ways across species as well (Cook, 1977; Hurtado-Gonzales et al., 2014; Spieth, 1952); in *D. bipectinata*, the combs are used to grasp the female and are pressed against either side of her abdomen prior to mating, potentially delivering tactile stimuli to the female and influencing her mating response. The size of the sex comb in *D. bipectinata* is condition dependent, heritable, known to be the target of pre-copulatory sexual selection in some natural populations, and larger than required for the mechanical function of grasping the female (Hurtado-Gonzales et al., 2014; Polak et al., 2015; Polak et al., 2004). Thus, the sex comb shares many characteristics with ornamental traits of animals generally (Andersson, 1994).

The physiological traits we contrasted between high and low selection lines in the present study include sperm length, number and viability, and testes and accessory gland size. Sperm viability was assayed both within the male seminal vesicle and the female seminal receptacle, the sperm storage organ within the female that houses the

sperm set most immediately deployed for fertilizations (Manier et al., 2010; Pitnick and Markow, 1994). A further dimension of our study investigates how these physiological traits may vary in relation to male age after eclosion, and respond to a key social variable, namely, presence of other sexually mature males. In *D. melanogaster*, exposure to potential competitors is known to influence expression of a variety of male postcopulatory traits (Bretman et al., 2009; Bretman et al., 2010; Wigby et al., 2009), including sperm viability (Moatt et al., 2014).

2. Methods

2.1. The base population and artificial selection protocol

The base population was established with 300 field-caught female *D. bipectinata* Duda (Diptera: Drosophilidae), and an approximately equal number of males captured from the surface of fruit substrates in Taiwan (25°2′30.24″ N, 121°36′39.37″ E). *Drosophila bipectinata* belongs to the *bipectinata* species complex (along with three other members, *D. parabipectinata*, *malerkotliana*, and *pseudoananassae*), within the *melanogaster* species group of the subgenus *Sophophora* (Bock, 1971).

The artificial selection protocol used to generate lines of *D. bipectinata* divergent in body-size specific comb size is described in detail elsewhere (Polak et al., 2021). Briefly, selection was applied for 11 consecutive generations in three replicate "high" lines (increasing comb size) and three replicate "low" lines (decreasing comb size) simultaneously. All lines were derived from the base population noted above, and which had been maintained in the laboratory for 4 generations prior to the onset of selection. Throughout the experiment, all lines were maintained in an environmental chamber under controlled light and temperature conditions (12 h light (24 °C):12 h dark (22 °C)).

Each generation of artificial selection, 105 males from each selected line ($n = 6$) were individually characterized in terms of thorax and comb size under an Olympus SZX12 stereomicroscpope (Olympus Corp., Center Valley, PA, USA). Thorax length (mm) was measured with an ocular micrometer, and comb size was determined by counting the number of individual teeth in both sex comb segments of each male, and averaged across the left and right legs. We regressed comb size on thorax length using general linear models (linear models were always appropriate), and the residuals were extracted and sorted in order of size. We applied selection for increasing comb size by choosing the 30 males as sires with the largest residual comb size to carry each generation forward, and selection applied for decreasing comb size by choosing the 30 males with the smallest residual comb size. Our protocol thus selected for sex comb size variation per unit body size, thus decoupling comb size from general body condition; body size in insects reflects nutritional history (i.e., condition) (Cotton et al., 2004; Emlen et al., 2012). The 30 selected males in each line were paired with 30 randomly chosen virgin females from within their respective lines, and cultured in bottles containing standard cornmeal-agar food.

2.2. Rearing flies from density-controlled larval environments

Post-selection, fly lines were maintained at a 12 h L (24 °C): 12 h D (22 °C) diurnal cycle in an environmental chamber. Test males used in the experiments were reared from density-controlled larval conditions. To do so, adult females and males from the 6 selection lines were used to seed grape-banana-agar oviposition medium. Hatched, first-instar larvae were transferred from the oviposition medium to vials (80 larvae per vial) containing standard cornmeal food in which larvae were allowed to develop through to adulthood.

2.3. Aging adult flies either singly or in groups

Upon eclosion, adult virgin flies were lightly anaesthetised with CO_2 and sorted by sex. Males were transferred to fresh cornmeal vials, either at a density of one male per vial (single housed) or 10 males per vial (group housed). All females were virgin individuals sourced from the original base population from which the selection lines were derived, and which was maintained in mass-culture over the course of selection. Females were aged in cornmeal vials with additional yeast, at a density of 10 flies per vial. Males were allowed to age for 4 – 9 days in cornmeal vials without extra yeast before use in the accessory gland and testis size experiment, or 4 – 8 days in all other experiments. Females were 4 – 8 days old. Males and females were transferred without anaesthesia to fresh cornmeal vials every 48 h. Each experiment was conducted such that all combinations of selection line, housing density and age were represented.

2.4. Testis length and accessory gland size

Flies were anaesthetised with ether fumes, transferred to a glass slide, and dissected with fine dissecting probes or biology-grade forceps in a drop of 1x phosphate-buffered saline (PBS) under an Olympus SZX12 microscope. In all cases the experimenter was blind to the identity of the male. The reproductive system was pulled out into the drop of PBS and one or both of the accessory glands, or paragonia (Gromko et al., 1984), transferred to a clean drop of saline, and one of the testis was transferred to a drop of paraffin oil and uncoiled (this process was found to be easier with paraffin oil than PBS). Digital images were captured using a SPOT Idea camera fitted to the microscope with SPOT v4.6 software (Diagnostics Instruments, Inc., Sterling Heights, MI, USA). Measurements were taken from these images using public domain ImageJ software (v1.37) (https://imagej.nih.gov/nih-image/). Accessory gland size was estimated as the area of the gland. This value was generated by the software from a line carefully traced around the entire perimeter of freshly dissected glands. If both glands were measured, a mean value was calculated. Testis length was measured by tracing a line through the centre of the testis, running its entire length (excluding the seminal vesicle). Testis length for 64 high (19, 24, 21 per line) and 60 low (20, 22, 18 per line) category males, and accessory gland area was determined for 64 high (22, 25, 17) and 61 low (20, 22, 19)_category males.

2.5. Sperm length

Each of the seminal vesicles was dissected into a drop of PBS on a slide coated in chrome alum-gelatin 'subbing' solution. The vesicle was gently punctured, allowing sperm cells to be released. The vesicle was then drawn through the PBS, allowing individual sperm cells to come free. Slides were oven-dried and imaged with an Olympus BX60 light microscope using Nomarski differential interference contrast (DIC). Images of individual sperm were captured using a Hitachi KP-F100 CCD Camera (Hitachi Denshi, Ltd., Japan) and Image-Pro Plus imaging software (Media Cybernetics, Silver Spring, MD, USA), and measured using ImageJ software (v1.37). We measured five sperm per male, from which we calculated a mean sperm length per male. Sperm length was measured for 45 (14, 15, 16 per line) and 46 (14, 14, 18 per line) high and low category males, respectively.

2.6. Sperm number

Each male was paired with a virgin female within a vial containing cornmeal food. Pairs were observed, and their times to initiate and terminate copulation recorded. Immediately after termination of copulation the female was removed, and dissected onto a subbed slide. The reproductive tract was pulled out into a drop of PBS. The uterus was transferred to a clean drop of PBS, punctured, and the sperm, which occurs as a single high-density mass, gently extracted. After discarding the uterine tissue, the ejaculate mass was teased apart with fine dissecting probes. The slide was oven-dried at 50 °C, and subsequently fixed in a methanol:acetic acid (3:1) solution, and stained with Hoescht 33258 which selectively tags DNA (Pitnick and Markow, 1994). Slides

were individually viewed using an Olympus BX60 light microscope under epifluorescence, and all sperm were counted twice independently, and the average sperm count calculated. Sperm number was quantified in the ejaculate of 59 (21, 17, 21) and 66 (23, 21, 22 per line) high and low category males, respectively.

2.7. Sperm viability within the male seminal vesicle

Each seminal vesicle (vas deferens) (Wolfner, 1997) was separated from the testis, punctured and sperm released into a 9 µl droplet of Beadle's saline solution. Causing as little damage as possible, sperm were gently teased out, and the seminal vesicle tissue discarded. To prevent evaporation, the dissections were encircled by a ring of Blu-Tack putty and covered with a second glass slide so that they were sealed within. Slides were transferred to a dark, humidified chamber and incubated for 50 min. After this time the top covering slide and putty were removed, and 4.5 µl dilute LIVE/DEAD sperm viability stain (Molecular Probes L-7011) added to each dissection. Dilute stain comprised 22 µl SYBR-14 and 44 µl of propidium iodide per 1 mL Beadle saline (Holman and Snook, 2008). A coverslip was placed on top of the preparation, and the slide placed back into the chamber for an additional 10 min incubation. Since sperm stored within the seminal vesicle have almost 100% survival (Holman, 2009a), the 1 h incubation time allows differences in sperm survival among groups to be exaggerated. Slides were viewed using an Olympus BX60 fluorescence light microscope fitted with a rhodamine filter at 330x magnification. As many sperm as possible in 10 min from each dissection were scored as alive (fluorescing green) or dead (red). Any sperm cells that fluoresced both green and red were scored as dead. Sperm viability scores were taken from 72 high (24, 23, 25 per line) and 75 low (24, 26, 25 per line) category males.

2.8. Sperm viability within the female seminal receptacle

Flies were mated as described in the sperm number protocol. Females were immediately isolated from males, and kept in groups on cornmeal food for 24 h before dissecting. The seminal receptacle (SR) was isolated from the reproductive system and transferred to Beadle's saline. Preparations were incubated, stained, and assessed for viability as above. Sperm viability scores were taken from 50 high (18, 16, 16 per line) and 45 low (17, 14, 14 per line) category males, respectively. Sperm for the viability assay was sourced from the female SR as this organ (as opposed to the paired spermathecae) is the most immediate source of fertilizing sperm (Manier et al., 2010).

2.9. Statistical analyses

Analyses were carried out using JMP® Pro 14.0.0 software (SAS, 2018). For each response variable we analyzed the data using a REML (restricted maximum likelihood) mixed model in which selection treatment ('high' or 'low'), age of the male, and housing category ('group' or 'single') were fitted as factors. Thorax length was included in all models as a covariate. In the case of sperm number transferred, copulation duration was included as a covariate also. For all traits, Line was nested within selection treatment and treated as a random effect. For each response variable, we started with a full model with all interaction terms included, and proceeded by sequentially removing non-significant interactions (P greater than 0.1) from the model in stepwise fashion. For sperm viability within the female SR and the male seminal vesicles, the data were expressed as a proportion (number viable sperm cells/total number examined), and arcsine-square root transformed. In the case of sperm viability within the female SR, because the three-way interaction between selection treatment, age and housing was significant, we retained all lower-order interaction terms in the final, reported model. Model residuals were normally or reasonably close to normally distributed for each response variable (testis length, Shapiro-Wilk $W = 0.979$, $P = 0.053$; accessory gland area, $W = 0.991$, $P = 0.65$; sperm length, $W = 0.984$, $P = 0.34$; sperm number, $W = 0.993$, $P = 0.764$; arcsine-sqrt proportion live sperm in female SR, $W = 0.982$, $P = 0.23$; arcsine-sqrt proportion live sperm in male vesicles, $W = 0.973$, $P = 0.0052$).

3. Results

Selection treatment did not have a significant effect on any male reproductive trait we examined (Table 1), with the exception of sperm viability within the female seminal receptacle (Table 2). For this sperm trait, the effect of selection treatment was complex, revealed by a significant three-way interaction between selection treatment, housing treatment and age (Table 2), which may be explained as follows. Among the males housed in groups (Fig. 1A), sperm viability of high line males increased with age, whereas for low line males, sperm viability tended to decrease with age. This differential effect of age between the selection categories resulted in high lines surpassing low line males in sperm viability within the female SR at age 5–6 and beyond (Fig. 1A). Among the males housed in groups, high line males overall had 8.4% higher sperm viability (mean (s.e.) proportion viable sperm, 0.734 (0.0523)) than low line males (0.677 (0.0620)).

Among males housed singly, there was an opposite pattern, where high line males tended (though weakly) toward having proportionately fewer viable sperm as they increased in age, whereas among low line males, viability trended upward with age (Fig. 1B). Among males held singly, high line males overall only had 2.6% greater sperm viability (0.703 (0.0572)) than low line males (0.685 (0.0659)).

Male age had a significant effect on a number of other sperm traits. Sperm length, sperm number within the ejaculate, and viability of sperm within the male's seminal vesicle, but not testis length, all showed significant positive relationships with male age (Tables 1 & 2, Fig. 2). For accessory gland size, there was also an effect of age, but this effect depended on housing treatment, revealed by a near-significant housing treatment-by-age interaction (Table 1): Males housed in isolation had a large gland area regardless of how old they were, whereas the glands of those housed in groups were lower when males were young, then

Table 1
Results of restricted maximum likelihood (REML) models examining responses in four male reproductive traits, from testis length to sperm number. Test statistics, degrees of freedom (df) and P values are provided. Significant parameters are highlighted in bold. Of the interaction terms, only those found to be significant (α < 0.05) or near significant are presented. Thorax length, entered as a covariate, was not significant in any case and was removed from all models. Line, nested within treatment, was treated as a random effect.

Trait	Factor	*F* statistic	df	*P*
Testis length	Selection treatment	0.535	1, 4.034	0.505
	Housing	0.083	1, 112.3	0.774
	Age	0.587	5, 112.6	0.710
	Line(Sel trt), Var component (s.e.) = 7.88e-3 (6.76e-3), Wald $P = 0.244$			
Accessory gland area	Selection treatment	5.205	1, 3.008	0.107
	Housing	**14.904**	**1, 112.6**	**<0.0002**
	Age	**3.142**	**4, 111.5**	**0.0172**
	Housing × age	**2.420**	**4, 111.3**	**0.0526**
	Line(Sel trt), Var component (s.e.) = -1.150e-5 (1.15e-5), Wald $P = 0.318$			
Sperm length	Selection treatment	0.0049	1, 4.324	0.947
	Housing	0.804	2, 79.55	0.451
	Age	**4.537**	**4, 78.79**	**0.0024**
	Line(Sel trt), Var component (s.e.) = -2.30e-4 (4.69e-4) , Wald $P = 0.624$			
Sperm number	Selection treatment	0.945	1, 5.462	0.372
	Housing	1.411	1, 117	1.411
	Age	**2.980**	**1, 114.9**	**0.022**
	Copula duration	**4.903**	**1, 116.2**	**0.029**
	Line(Sel trt), Var component (s.e.) = -1.468e3 (1.07e3), Wald $P = 0.169$			

Table 2
Results of restricted maximum likelihood (REML) models examining responses in sperm viability sampled from within the male seminal vesicles and the female seminal receptacle. Proportion viable sperm data in each case were arcsine-square root transformed prior to analysis. Thorax length, entered as a covariate, was not significant in any case and removed from both models. Test statistics, degrees of freedom (df) and *P* values are provided. Significant parameters are highlighted in bold. Line, nested within treatment, was treated as a random effect.

Trait	Factor	*F* statistic	df	*P*
Sperm viability in males	Selection treatment	0.748	1, 3.948	0.436
	Housing	0.0842	1, 136.5	0.772
	Age	**5.732**	**4, 137.6**	**0.0003**
	Line(Sel trt), Var component (s.e.) = -1.85e-4 (1.0e-3), Wald $P = 0.853$			
Sperm viability in females	Selection treatment	0.826	1, 3.365	0.424
	Housing	0.178	1, 74.75	0.674
	Age	1.185	4, 73.4	0.325
	Sel trt * Housing	0.0302	1, 74.75	0.862
	Sel trt * Age	0.562	4, 73.4	0.691
	Housing * Age	0.664	4, 72.925	0.619
	Sel trt × Housing × Age	**3.00**	**4, 72.92**	**0.024**
	Line(Sel trt), Var component (s.e.) = -2.10e-3 (3.0e-3), Wald $P = 0.483$			

increased with age (Table 1, Fig. 3).

Sperm viability was significantly positively related to total number of sperm within the female seminal receptacle (coefficient (SE) = 0.00242 (0.000435), $t_{89} = 5.57$, $P < 0.0001$) (Fig. 4), but for sperm within the male seminal vesicle, this relationship was not significant (coefficient (SE) = 8.194e-5 (8.749e-5), $t_{141} = 0.937$, $P = 0.351$) (Fig. 4). Sperm viability was found to be significantly higher within the male seminal vesicle (mean (SE), 0.557 (0.0187)) than within the female seminal receptacle (0.456 (0.0242), $F_{1,223.8} = 7.525$, $P = 0.0066$).

4. Discussion

We tested for correlated changes in male postcopulatory traits in response to artificial selection on male sex comb size in *D. bipectinata*. The sex comb is a heritable secondary sexual trait known to be under precopulatory sexual selection in some natural populations of *D. bipectinata*, and to be undergoing incipient evolutionary diversification both within and between closely related taxa (Bock, 1971; Bock and Wheeler, 1972; Mishra and Singh, 2006; Polak and Starmer, 2005; Polak et al., 2004). The present study was motivated by previous research indicating a positive genetic correlation between sex comb size and male competitive fertilization success, concomitant with upregulation of specific seminal fluid proteins (SFPs) in the large-combed genetic lines (Polak et al., 2021). Our approach here was to contrast these lines divergent in sex comb size generated by artificial selection in respect to a suite of complementary physiological traits, with the intent of gaining a deeper understanding of the causal factors that may underlie the observed correlated divergence in competitive fertilization success between high and low sex comb lines.

With the notable exception of sperm viability within the female's seminal receptacle (SR), we found no significant correlated response to selection on comb size in any of the post-copulatory traits we examined. Interestingly, this significant effect on sperm viability in the SR was manifested as a three-way interaction between selection treatment, housing treatment and age, such that the sperm viability of high line males held in groups only surpassed that of low line males when they were between 5 and 6 days old and older. This complex interaction, however, occurred only for sperm stored within the female seminal receptacle; it was not observed for sperm sampled from the male seminal

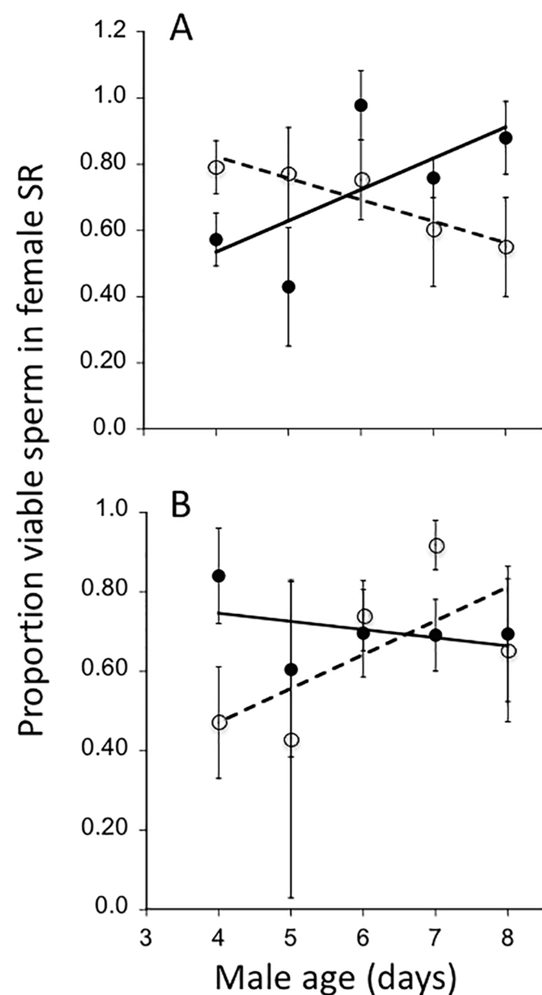

Fig. 1. Proportion live sperm (arcsine(square root) - transformed) within the female seminal receptacle (SR) 24 h post-mating, across male age at mating. **(A)** Males housed in groups; **(B)** Males housed singly. Solid lines represent high-line males, broken lines represent low-line males in both panels.

vesicle, which is consistent with upregulated SFPs in the large-combed lines (Polak et al., 2021). In *Drosophila*, spermatogenesis occurs within the testes, and mature sperm cells are stored within the seminal vesicles; sperm are then combined with secretions of the accessory glands within the anterior ejaculatory duct before ultimately being transferred to the female across the ejaculatory bulb (Gromko et al., 1984; Wolfner, 1997). Thus, assuming that SFPs affect sperm viability (see below), we would not have expected to see any effects of genetic line on sperm viability sampled from the male seminal vesicles, since sperm in this organ have yet to be blended with SFPs.

Seminal fluid components are known to have positive effects on sperm viability in *D. melanogaster* (Holman, 2009a), as well as in other insects, including bees (King et al., 2011) and ants (den Boer et al., 2008; Dosselli et al., 2019). Notably, one of the SFPs (Acp62F) that was found upregulated in our high sex comb lines (Polak et al., 2021) localizes to the female seminal receptacle in *D. melanogaster* (Lung and Wolfner, 1999), suggesting a role of this gene in sperm maintenance (Ram and Wolfner, 2007). However, whether Acp62F modulates sperm viability or other quality parameters is not known, and other functions of this protein have been identified (Lung et al., 2002). In general, SFPs could affect sperm viability in different ways, for example, by protecting sperm from oxidative damage or a female immune response, or in the differential maintenance of live sperm in storage within the female (Holman, 2009a). In this light, SFP-mediated effects on sperm survival

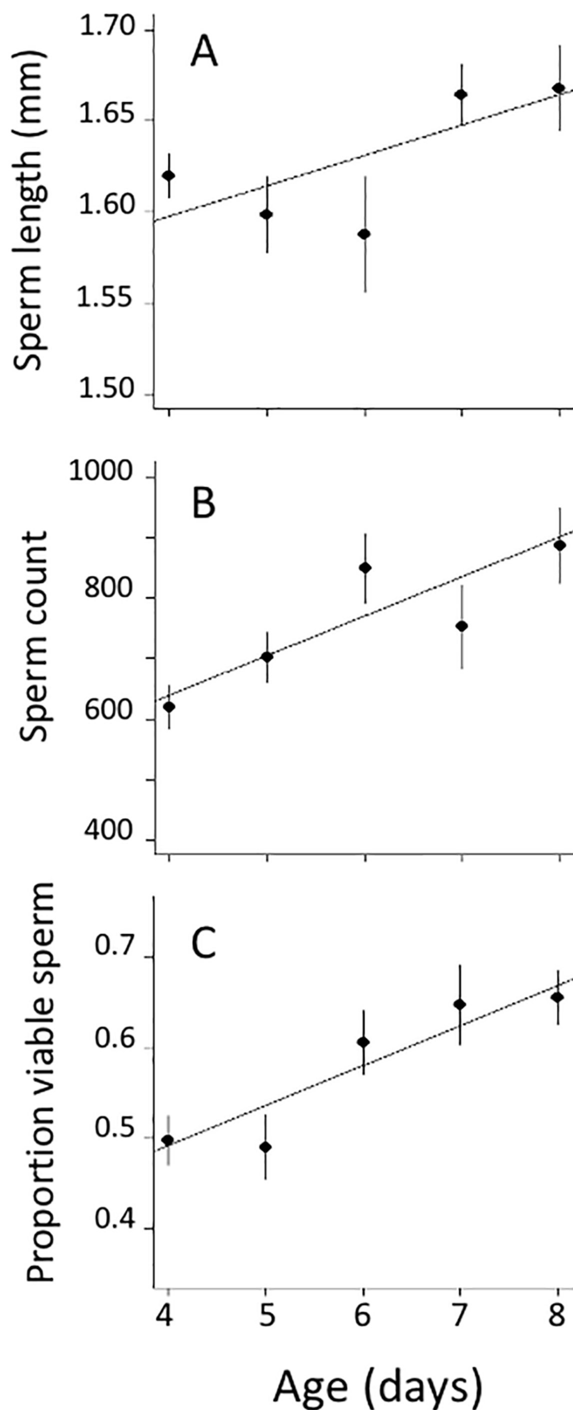

Fig. 2. Increases in male traits with age (means ± 1 S.E.). (**A**) Sperm length (mm); (**B**) Number of sperm within an ejaculate; (**C**) Raw proportion of live sperm within the male seminal vesicle.

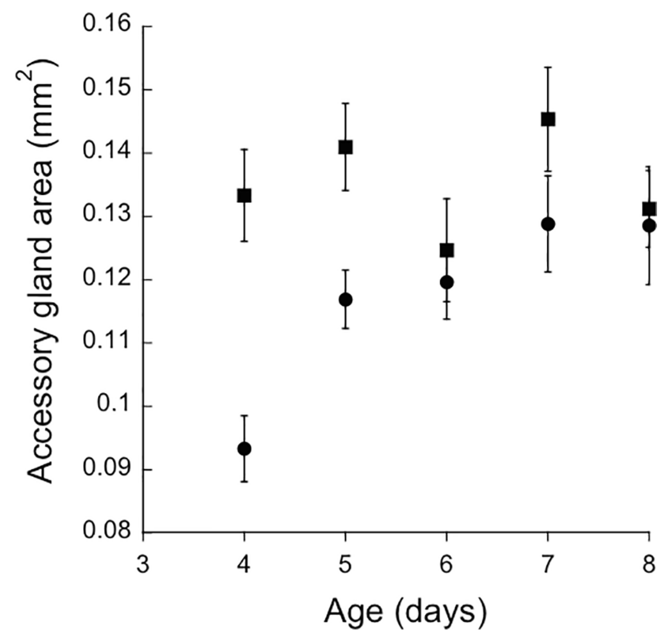

Fig. 3. Accessory gland size as area (mm^2) across male age (means ± 1 S.E.). Circles show males housed in groups. Squares show males housed in isolation.

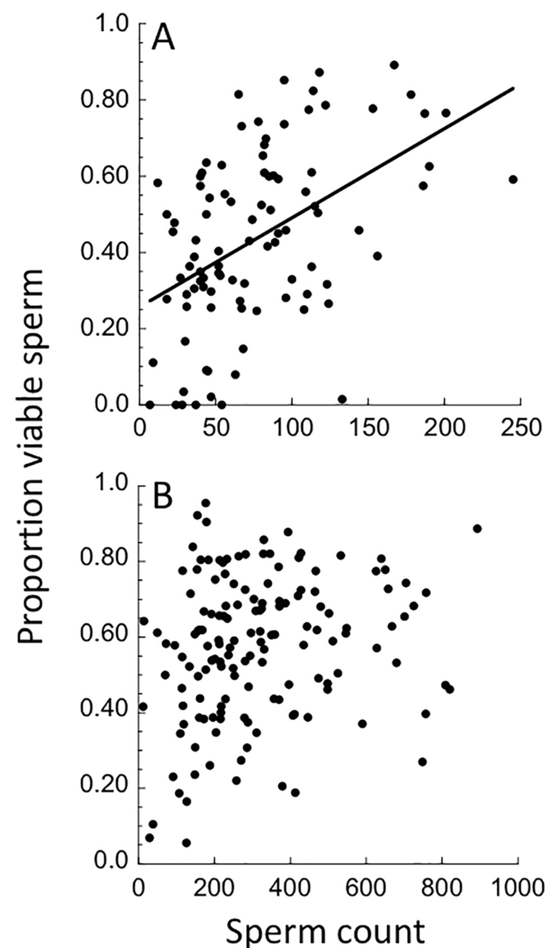

Fig. 4. Relationship between the proportion sperm alive and total number of sperm sampled from (**A**) the female seminal receptacle (SR); and (**B**) the male seminal vesicle.

might also underlie the observed relationship between sperm number and viability for sperm from the female SR but not the male seminal vesicle.

It should be emphasized that the study by Polak et al. (2021) assayed competitive fertilization success of males that, prior to mating trials with previously inseminated females, had been housed under conditions equivalent to our 'group' treatment, but that were younger, generally ranging in age from 3 to 4 days at experimental mating. It is therefore plausible, but by no means strong evidence owing to the age mismatch, for the viability of sperm stored within the female to have contributed to the heightened fertilization success enjoyed by high line males.

Moreover, while Polak et al. (2021) measured fertilization success in the context of sperm competition, in the present study we only examined viability of sperm within singly mated females, and we are unsure of how the presence of a competitor's ejaculate might affect sperm viability (e.g., (Holman, 2009a)).

Another relevant issue with sperm viability studies is that sperm may be killed through the process of handling and staining (Holman, 2009b; Stewart et al., 2007), potentially compromising the accuracy of such assays seeking to estimate absolute numbers of living sperm within a given sample. In the present study, we sought differences in viability among similarly handled treatment groups rather than absolute estimates, so our approach using this technique should be valid (Holman, 2009b). However, the potentially reduced sensitivity of the assay means that any real but subtle differences among groups at younger ages may have been missed.

It is surprising that we did not find an effect of selection treatment on sperm number within the ejaculate, since sperm number is expected and often found to affect competitive fertilization success in insects (Simmons, 2001). It is possible that we were unable to detect it due, again, to a shortcoming of the assay. The relatively long sperm of *D. bipectinata* (1.63 mm) (this study) is similar in length to *D. melanogaster* (1.85 mm) (Manier et al., 2013), and the sperm mass can be challenging to untangle. Even with very little handling, the single sperm mass can form dense clumps through which the fluorescent heads can be difficult to count. Relatively minor, stochastic differences in degree of sperm clumping could lead to counting errors. The protocol was, however, able to detect differences in sperm numbers among age categories. This suggests that, despite these practical issues, our methods were sensitive enough to detect an effect of selection treatment on sperm number had one existed.

The effects of male age on the physiological traits we examined were more pervasive than effects of selection treatment, which, as described above, was restricted to an effect on sperm viability within the female seminal receptacle. We detected effects of male age on sperm length and number within the ejaculate, as well as the proportion viable sperm with the male seminal vesicle. Our finding of an increase in sperm length with age is interesting, as this is not a commonly reported phenomenon, and in *Drosophila melanogaster* sperm length appears to be insensitive to other factors such as male nutritional history and physiological condition (Lüpold et al., 2016). However, increases in sperm length with age have been documented in other species, for example, in a warbler, *Setophaga caerulescens* (Cramer et al., 2020), guppies, *Poecilia reticulata* (Gasparini et al., 2010), and a rove beetle, *Aleochara bilineata* (Green, 2003). Noteworthy also is that we found this increase in sperm length with age in the absence of a detectable increase in testis length. This lack of a concomitant increase in testis length may not, however, be surprising, given that the youngest males in our study were already 4 days of age. At 4 days of age males would have fully grown testes, so that no further increase with age would be expected although sperm length continued to increase. Male *D. bipectinata* reach sexually maturity at $\leq$ 1–2 days of age post-emergence, not dissimilar to *D. melanogaster* (Manier et al., 2013; Pitnick, 1996; Ruhmann et al., 2016), and so we would expect any growth in testis length to have stopped at approximately 24 h post emergence. By contrast, in *D. pachea*, a remarkable species whose sperm length is more than 10 times that of *D. bipectinata*, testes require about 12 days to grow and fully elongate, and consequently, males require 12–16 days to start producing mature sperm and reach mating readiness (Pitnick, 1993).

We also found sperm viability scores to be higher for older males than for younger males for sperm sampled from the male seminal vesicle. This finding suggests that the accumulation of sperm in older males affords some form of protection. Since these sperm were taken from the male seminal vesicle, this age-related protection is unlikely to be conferred by seminal fluid components, but rather, by other factors specific to the testicular or seminal vesicular environments (Holman, 2009b). Effects of age on sperm viability have also been found in the Australian field cricket, *Teleogryllus oceanicus* (Garcia-Gonzalez and Simmons, 2005), and *D. melanogaster* (Decanini et al., 2013). In the latter case, older males were found to store more sperm within their seminal vesicles, and that these sperm, despite having been stored for a longer period of time as reported here, were more viable.

We might have also predicted an increase in sperm number associated with males housed in groups, as has been shown in *D. melanogaster* (Garbaczewska et al., 2013; Lüpold et al., 2011; Moatt et al., 2014). Prolonged exposure to other males could encourage greater quantitative investment of sperm into ejaculates, in response to sperm competition risk (Bretman et al., 2010; Wedell et al., 2002). That we did not find a relationship suggests lack of a history of selection for a plastic response in sperm number to social cues in our focal species, or that the presence of other males for the time duration in our study was not sufficient to induce increased investment in this sperm trait (Bretman et al., 2010).

Our observation that accessory gland size increased with age could be the result of holding the males as virgins for a prolonged period of time, allowing accessory gland products to accumulate, and the gland to increase in size (Herndon et al., 1997; Rogers et al., 2005b). If this were the case, then we would expect the glands of males in both housing treatments to have similarly increased with time. However, this increase was only seen in males that had been housed in groups. In contrast, of those housed in isolation, their accessory glands were consistently large. This instead suggests that males housed in the high-density environment may have taken longer to reach the optimal gland size achieved by their single-housed counterparts, possibly due to the physiological/energetic costs associated with social interaction. Indeed, accessory gland size does appear to carry physiological costs, for example, replenishment of gland products after copulation may take up to 24 h in *D. melanogaster*, and up to 48 h in stalk-eyes flies, *Cyrtodiopsis dalmanni* (Rogers et al., 2005b). In *C. dalmanni*, the development of larger accessory glands delays the onset of sexual maturity, and accessory gland size is reduced in response to increases in testis size due to meiotic drive (Meade et al., 2020), suggesting a trade-off between accessory gland size and testis size in this species, and further indicating that costs associated with accessory gland investment may be a general phenomenon.

Previous work with both *D. bipectinata* (Santhosh and Krishna, 2013) and *D. melanogaster* Decanini et al. (2013) has also found that older males possess larger accessory glands. In *D. melanogaster*, Ruhmann et al. (2016) also documented a progressive increase in accessory gland size with age for males 1 to 6 days old, and showed that this increase was accompanied by both improved sperm competitive ability (as defensive ability, P_1, in this case) and decreased female remating propensity, implying increased SPF production and transfer with age. It should be noted that, although we also identified differences among males in accessory gland size, this would not necessarily translate into differential transfer of specific SFPs at mating, as it apparently cannot be assumed that gland size consistently predicts different SFPs in terms of quantity produced. For example, Wigby et al. (2009) selected for large and small accessory gland size in *D. melanogaster*, and despite larger resultant glands in the up-selected lines, males in these lines had increased quantities of sex peptide but not ovulin. In another study of the same species, gland size did not correlate with sperm displacement ability ((Bangham et al., 2002)), despite the fact that gland size was positively related to another reproductive parameter, mating frequency, as also shown in *C. dalmanni* (Rogers et al., 2005a,b). Further genetic and physiological analyses relating sexual signalling to accessory gland products and the fertilizing efficiency of ejaculates are needed in these and other systems.

Author statement

F.T. conducted and supervised the research, contributed to data analysis and interpretation, and the writing of the original draft of the manuscript. S.H. and A.I. conducted experiments, physiological assays, data entry and curation. M.P. acquired funding for the research,

supervised the research, contributed to data analysis and interpretation, and the contributed to writing, review and editing of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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