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RESEARCH ARTICLE

Sexual selection drives the coevolution of male and female reproductive traits in *Peromyscus* mice

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Abstract

When females mate with multiple partners within a single reproductive cycle, sperm from rival males may compete for fertilization of a limited number of ova, and females may bias the fertilization of their ova by particular sperm. Over evolutionary timescales, these two forms of selection shape both male and female reproductive physiology when females mate multiply, yet in monogamous systems, post-copulatory sexual selection is weak or absent. Here, we examine how divergent mating strategies within a genus of closely related mice, *Peromyscus*, have shaped the evolution of reproductive traits. We show that in promiscuous species, males exhibit traits associated with increased sperm production and sperm swimming performance, and females exhibit traits that are predicted to limit sperm access to their ova including increased oviduct length and a larger cumulus cell mass surrounding the ova, compared to monogamous species. Importantly, we found that across species, oviduct length and cumulus cell density are significantly correlated with sperm velocity, but not sperm count or relative testes size, suggesting that these female traits may have coevolved with increased sperm quality rather than quantity. Taken together, our results highlight how male and female traits evolve in concert and respond to changes in the level of post-copulatory sexual selection.

KEYWORDS
cryptic female choice, mammals, sexual conflicts, sexual selection, sperm competition

1 | INTRODUCTION

In sexually reproducing species, each sex influences the social environment, and thus the selective regime, for which the other sex evolves (Svensson et al., 2019). In polyandrous systems, in which females mate with multiple males within a single reproductive cycle, the female reproductive tract of internally fertilizing species can become an arena of competition and conflict. Sperm competition theory predicts that ejaculates from rival males compete for fertilization of available ova (Parker, 1970), yet sexual conflict can arise if competitive male strategies reduce female reproductive optima (Hosken et al., 2019). Therefore, post-copulatory sexual selection is also expected to favour mechanisms that enable females to bias sperm use and prevent polyspermy, termed cryptic female choice (Eberhard, 1996; Firman et al., 2017). In species with multiple mating, these powerful forms of selection are hypothesized to drive the evolution of traits that enable males to compete for fertilization and females to exert control after mating has occurred; monogamous species, however, are expected to experience weak post-copulatory sexual selection (Kvarnemo, 2018; Parker, 1984). Here, we examine

how divergent mating systems within the rodent genus *Peromyscus* have shaped the evolution of reproductive traits in both sexes.

One of the primary adaptations of sperm competition is to increase the number of highly motile sperm, which is often associated with larger, more productive testes (reviewed in Lüpold et al., 2020). Only a small fraction of sperm survive migration through the female reproductive tract in internally fertilizing species (Kunev, 2019), and when females mate with more than one male, males who produce relatively more sperm are predicted to experience a reproductive advantage if a greater number of their sperm survive to the fertilization site (Parker & Pizzari, 2010). Sperm production is regulated by Sertoli cells, which provide nutrients and scaffolding to the developing germ cells within the seminiferous tubules (França et al., 2016; Griswold, 2018). Higher concentrations of Sertoli cells positively correlate with sperm count (Pintus et al., 2015) and can lead to larger testes (França et al., 1995). As predicted by theory (Parker & Pizzari, 2010), relative testes weight is associated with level of sperm competition across a range of diverse taxa (reviewed in Lüpold et al., 2020; Simmons & Fitzpatrick, 2012) including rodents (Ramm et al., 2005). While investment in overall testes size may be the most common adaptation to changes in sperm competition intensity, or at least the best studied, changes in spermatogenic rate and testicular architecture can also affect sperm production (del Barco-Trillo et al., 2013; Ramm & Schärer, 2014) and, thus, be targets of sexual selection. For example, an interspecific comparison across mammals revealed that testes of promiscuous species exhibit greater seminiferous tubule density than those of monogamous species (Harvey & Harcourt, 1984), and in an experimental evolution study, multiply mated lines of mice (*Mus musculus*) exposed to high levels of sperm competition exhibited testes greater density of seminiferous tubules and higher sperm counts than the monogamous lines (Firman et al., 2015). In addition to influencing sperm production, seminiferous tubule density may also impact other sperm traits. For example, seminiferous tubule density is positively associated with sperm length, a trait frequently correlated with sperm velocity (Gomendio & Roldan, 2008; but see Lüpold et al., 2009). Faster sperm are expected to reach the fertilization site earlier when ejaculates from multiple males co-occur within the female reproductive tract, and across many taxa, sperm velocity is higher in systems with multiple mating (Snook, 2005).

Traits that improve male reproductive success in competitive environments can benefit females if they promote fertilization by sperm from the highest quality males (Firman et al., 2017). However, many traits favoured by post-copulatory sexual selection in males have a negative impact on female viability, reproductive output or their ability to control the paternity of their offspring (Eberhard, 1996; Firman et al., 2017). For example, an increase in the number of highly motile sperm that reach the fertilization site increases the risk of polyspermy, which can stop embryo development in many systems (Hunter, 1996). Mechanisms of female control can reduce opportunities for polyspermy and fertilization by suboptimal sperm (Firman et al., 2018; Franke et al., 2002; Levitan et al., 2007). The fluidic environment of the female reproductive tract, for example, can modulate sperm performance via changes in pH and viscoelasticity (Tung & Suarez, 2021), and expose sperm to extracellular vesicles and immune cells that treat them as foreign invaders (Rickard et al., 2019; Suarez & Pacey, 2006). In many mammals, cumulus cells that support oocyte maturation also impede sperm access, therefore, sexual selection may favour large cumulus masses in females and the fertilization by sperm that release hyaluronidase, an enzyme that disassociates the cumulus cells from the oocytes. In internally fertilizing species, the shape of the female reproductive tract can also influence fertilization by diverting or storing sperm, and increased length can promote fertilization by the most motile and long-lived sperm (reviewed in Holt & Fazeli, 2016). Although mechanisms of post-copulatory female control are less studied than male traits, they can have profound consequences on reproductive success and, thus, are important targets of sexual selection (Orr et al., 2020).

Closely related species that have evolved under divergent mating systems can provide insight into how sexual selection has shaped the evolution of reproductive traits, and mice in the genus *Peromyscus* display a wide range of natural mating strategies (Bedford & Hoekstra, 2015). While most *Peromyscus* species are promiscuous (i.e. both males and females mate multiply), monogamy has evolved independently at least twice (Turner et al., 2010). In this study, we examine three promiscuous species (*P. maniculatus*, *P. leucopus* and *P. gossypinus*) that are predicted to have evolved under intense post-copulatory sexual selection, and three monogamous species, in which post-copulatory sexual selection is relaxed. Two of the monogamous species, *P. californicus* (Ribble & Salvioni, 1990) and *P. polionotus* (Foltz, 1981), form pair bonds, produce single paternity litters, and are biparental. In contrast, in *P. eremicus*, monogamy is thought to result from limited access to mates and, therefore, it is considered facultatively monogamous (Eisenberg, 1963). Captive breeding of these six species with distinct evolutionary histories in nearly identical conditions and social environments, including enforced monogamy (Bedford & Hoekstra, 2015), allows for controlled experiments to compare differences in reproductive traits associated with divergent mating systems. Here, we examine quantitative variation in reproductive traits among species to test two hypotheses. First, that sperm competition drives the evolution of traits that increase sperm production and sperm swimming performance in species that have evolved under a promiscuous mating strategy compared to monogamous species. Second, that selection favours mechanisms that allow female control of fertilization in systems where females mate with multiple males. Following these hypotheses, we predict that reproductive traits associated with male competition for access to oocytes and female control of fertilization will be correlated across species with divergent evolutionary histories in *Peromyscus* mice.

2 | METHODS

2.1 | Experimental animals

We obtained captive strains of *P. californicus insignis*, *P. eremicus*, *P. polionotus subgriseus*, *P. maniculatus bairdii* and *P. leucopus* from the *Peromyscus* Genetic Stock Center (University of South Carolina)

and *P. gossypinus* from Dr. Hopi Hoekstra (Harvard University). We reared all mice to >100 days old at 22°C under a 16L:8D light cycle at the University of Maryland in accordance with guidelines established by the Institutional Animal Care and Use Committee (protocol R-Jul-18-38). We avoided including wild-caught mice in our dataset to reduce variation due to resource availability and social and reproductive experience, which can have a profound influence on reproductive traits (Stearns, 1992). Some samples included in this study were collected opportunistically with other experiments, thus not all traits were measured on all animals. However, testes and body weight were obtained for all males, and body weight and length (snout to vent) were obtained for all females. The final dataset includes 405 animals: 47 *P. californicus* males and 19 females, 27 *P. eremicus* males and 22 females, 46 *P. polionotus* males and 32 females, 45 *P. maniculatus* males and 33 females, 40 *P. leucopus* males and 31 females and 35 *P. gossypinus* males and 28 females. Prior to tissue harvest, we euthanized subjects with isoflurane overdose, followed by cervical dislocation.

2.2 | Male reproductive traits

We obtained all samples from sexually mature virgin males, except for *P. eremicus*, which required pairing with a female to produce viable sperm (see Methods S1). We controlled for differences in sexual experience and age, since sexual maturity varies by species, in all statistical analyses. We weighed each male, then removed and weighed both testes from each subject, and calculated relative testes weight by dividing by body weight. To collect live sperm, we excised the left caudal epididymis, made three incisions, and incubated the tissue at 37°C for 1 h with 300 rpm agitation in 1000 µl of Modified Human Tubal Fluid (mHTF; Irvine Scientific) supplemented with 5 mg/ml of Probumin bovine serum albumin (Millipore Sigma).

To estimate sperm count ($N = 15$ for all species, except $N = 10$ for *P. leucopus*), we collected live sperm after the one-hour incubation, inverted the tube of suspended sperm three times to homogenize, then pipetted 95 µl into another tube containing 5 µl of formalin, pipetted gently to mix and aliquoted 10 µl onto a haemocytometer (Marienfeld Company). We imaged five haemocytometer grids at 250× magnification using an AxioCam 105c camera on an AxioPlan microscope (Carl Zeiss AG), then used ImageJ (version1.53a, [Schindelin et al., 2012]) to estimate sperm count following the World Health Organization guidelines (WHO, 2010; Methods S1).

To estimate seminiferous tubule density ($N = 15$ for all species), we submerged the left testis in Bouin's fixative (Sigma), dehydrated it using an ethanol series, then cleared the tissue in Histo-Clear (National Diagnostics) prior to embedding in paraffin wax, sectioned midsagittally at 4 µm and stained sections with haematoxylin and eosin (Methods S1). We imaged eight regions on each section unassociated with the mediastinum at 100× magnification using an AxioCam 105c camera on an AxioPlan microscope (Carl Zeiss AG) and used the curvature pen tool in Adobe Photoshop CC (version 19.1.14) to outline the basal membrane of each imaged seminiferous tubule and the fill path tool to mask the tubule, including the lumen, revealing only the interstitial space. We then used ImageJ to quantify the area of each masked image to estimate the seminiferous tubule density and calculated the mean across all eight regions sampled per individual.

To estimate sperm velocity ($N = 15$ for all species), we loaded 12 µl of suspended sperm into an 80 µm chamber slide (Cell-Vu) and used a computer-assisted sperm analysis system (CEROS II, Hamilton Thorne) to analyse 10 five-second videos per male. We excluded all immotile sperm from the analysis and report sperm curvilinear velocity here for simplicity (but see other kinematic parameters in Table 1).

2.3 | Female reproductive traits

We obtained samples from virgin females during the prooestrus phase of the oestrous cycle, which we monitored following

TABLE 1 Mean and SD (standard deviation) of sperm kinematic parameters across *Peromyscus*

	Monogamous *Peromyscus*			Promiscuous *Peromyscus*		
	californicus	*eremicus*	*californicus*	*maniculatus*	*leucopus*	*gossypinus*
DAP (µm)	60.04 (16.45)	56.07 (13.81)	68.18 (23.31)	78.19 (27.74)	81.69 (19.11)	51.54 (16.38)
DSL (µm)	47.70 (14.41)	43.54 (12.60)	51.27 (24.04)	60.04 (21.40)	57.07 (14.46)	40.60 (15.54)
DCL (µm)	111.33 (28.16)	91.12 (17.29)	109.14 (24.90)	137.06 (34.60)	141.93 (28.95)	104.28 (25.87)
VAP (µm/s)	62.25 (15.09)	53.70 (13.08)	72.05 (23.05)	88.70 (23.62)	70.63 (19.05)	58.57 (19.73)
VSL (µm/s)	48.84 (13.18)	42.43 (11.95)	56.58 (23.82)	70.02 (21.40)	52.08 (16.43)	46.20 (18.94)
VCL (µm/s)	116.21 (26.08)	88.57 (16.90)	116.55 (25.16)	159.58 (36.70)	121.72 (29.07)	115.70 (29.76)
STR	73.53 (15.52)	70.92 (10.87)	72.75 (19.98)	73.39 (13.71)	71.63 (9.34)	72.68 (20.73)
LIN	41.37 (9.15)	44.05 (9.14)	46.28 (18.28)	43.32 (10.25)	41.92 (9.11)	39.14 (13.59)
WOB	53.59 (10.28)	57.14 (10.50)	59.97 (20.75)	55.22 (10.13)	56.89 (6.87)	50.08 (14.04)

Note: Kinematic values shown include length of average path (DAP), length of straight-line path (DSL), length of curvilinear path (DCL), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), straightness (STR) calculated by VSL/VAP, linearity (LIN) calculated by VSL/VCL and wobble (WOB) calculated by VAP/VCL.

methods in Byers et al. (2012). To collect samples, we removed the ovarian bursa and separated the ovaries from the rest of the reproductive tract.

To extract cumulus-oocyte complexes (COC; $N = 15$ for all species), we excised all Graafian follicles, placed them in mHTF and ruptured the follicle using the tip of a 27-gauge needle. We then allowed the COC to passively release from the follicle and imaged at 200× magnification using an Axiocam ICc5 camera on a Stemi 508D microscope (Carl Zeiss AG). We used the freehand tool in ImageJ to estimate the two-dimensional area, then calculated the mean area for each individual. This provided a spatial estimate of the cumulus mass surrounding the oocytes, but because the number of cumulus cells within a complex can vary, we also measured the relative number of cumulus cells released by the ovaries per oocyte. To estimate the number of cumulus cells, we incubated all COCs per individual in 10 μl of type IV-5 Bovine Testes Hyaluronidase (Millipore Sigma) for 15 min to dissociate cells, inverted the tube to homogenize, increased the volume to 500 μl with mHTF, inverted again and used a Sceptre (Millipore Sigma) to count cells. To calculate relative number of cumulus cells per oocyte, we divided cumulus cell count by total number of oocytes released for each female.

To measure oviduct length ($N = 15$ for all species), we severed each uterine horn near the uterotubal junction and carefully removed the broad ligament to prevent stretching of the tissue. We then gently straightened each oviduct, covered it with a cover slip to flatten the tissue and imaged at 6.2× magnification using an Axiocam ICc5 camera on a Stemi 508D microscope (Carl Zeiss AG). In ImageJ, we measured the length of the oviduct, from the edge of the infundibulum to the edge of uterotubal junction, but not including either the infundibulum or the uterotubal junction in the measurement, using the segmented line tool and calculated the mean oviduct length for each female, then calculated relative oviduct length by dividing by body length.

2.4 | Statistical analyses

We conducted all statistical analyses in R (version 3.5.1; R Foundation, 2018) within the RStudio environment (Version 1.1.463; RStudio Team, 2015). To examine if reproductive traits differed by species, we conducted separate linear models (LM) for each trait. As age, sexual experience and overall body weight can be positively associated with sperm count, sperm velocity and seminiferous tubule density (reviewed in Hook & Fisher, 2020), we compared models for each of these reproductive traits with and without age, sexual experience and body weight as covariates using Akaike Information Criterion (AIC; Burnham & Anderson, 2004) and determined the best fitting model for each trait by the lowest AIC value. For sperm count, the best-fitting model included only age as a covariate. For seminiferous tubule density and sperm velocity, the best-fitting models included age and body weight as covariates. We conducted a similar analysis for the female reproductive traits, yet since all females were virgins, we considered only age and body weight for cumulus cell area and cumulus cell count per oocyte models. The best-fitting model for cumulus cells per oocyte included body weight as a covariate, and for cumulus cell area, age was a covariate. Due to size differences between the species, we found that modelling relative testes weight and relative oviduct length, rather than raw measures with body size as a covariate, yielded lower AIC values, but otherwise, these models considered the same covariates as other male and female trait LMs. The best-fitting model for relative testes weight and relative oviduct length included age as covariates. Finally, we standardized the models to a *y*-intercept of zero (Beckerman et al., 2017).

To examine how reproductive traits differ by mating system, we first used separate models within a phylogenetic generalized least squares (PGLS) framework (Freckleton et al., 2002) using the 'caper' (Orme et al., 2018) and 'APE' (Paradis et al., 2004) R packages for each trait. In this analysis, we used an ultra-metric *Peromyscus* phylogenetic tree based on sequence variation in cytochrome B, and matched species' relationships from other previously established phylogenies of *Peromyscus* (Bradley et al., 2007; Turner et al., 2010). The models included sperm count, relative testes weight, seminiferous tubule density, sperm velocity, COC area, cumulus cells per oocyte or relative oviduct length as response variables, and mating system (monogamous or promiscuous) as the main predictor but also considered age, body weight and sexual experience for male traits, and age and body weight for female traits but excluded sexual experience since all females were virgins. We again found that modelling relative testes weight and relative oviduct length yielded the best PGLS models, therefore we excluded body weight in these models. The final models for each trait yielded a Lambda score of 0, suggesting that there was phylogenetic signal in every trait, however the PGLS framework is less reliable when relatively few species are compared and when models include binary variable (Garamszegi & Møller, 2010; e.g. mating system), therefore, so we also conducted separate linear mixed effects models (LMM) for each trait using the 'lme4' package in R (Bates et al., 2015) to compare reproductive trait differ by mating system without controlling for phylogeny. For sperm count, sperm velocity and seminiferous tubule density models, we considered mating system, age, body weight and sexual experience as fixed effects and species as a random effect. We considered the same fixed and random effects for cumulus cell area and cumulus cell count per oocyte models but excluded sexual experience as a fixed effect since all females were virgins. We again found that modelling relative testes weight and relative oviduct length yielded the best LMM, therefore we excluded body size as a fixed effect in these models, otherwise these models considered the same fixed and random effects of other male and female trait LMM. For each analysis, we report the best-fitting model as determined by the lowest AIC value. The best-fitting models for sperm count and seminiferous tubule density included mating system, body weight. and age as fixed effects, the model for relative testes weight included mating system, age, sexual experience and for sperm velocity the model included mating system and sexual experience as fixed effects. The best-fitting model for COC area and relative

oviduct length only include mating system as a fixed effect, and the best-fitting model for cumulus cells per oocyte included mating system and age as a fixed effect.

We next examined correlations between traits while accounting for phylogenetic relatedness among species within a PGLS framework for each trait. To understand the association between male and female traits across species, we compared the species mean of each male trait to the species mean of each female trait to estimate the Pearson's product moment correlation coefficient within this PGLS framework to account for species relatedness. Next, we performed an interspecific correlation analysis within a PGLS framework to compare each female trait to other female traits, and each male trait to other male traits, to investigate, for example, if relative testes size correlates with sperm count, or if relative oviduct length correlates with the number of cumulus cells per oocyte across species.

In addition to the cross-species comparisons, we examined intraspecific variation of testes weight to sperm count, seminiferous tubule density and sperm velocity using Pearson's product–moment correlations. Due to our sampling methods, we did not have sufficient data from multiple traits of the same individuals to perform additional within-species comparisons.

3 | RESULTS

We found all reproductive traits recorded in this study differed significantly between all focal *Peromyscus* species, except for number of cumulus cells per oocyte in the three monogamous species (Tables 2 and 3).

We found that the focal promiscuous *Peromyscus* species exhibited significantly greater sperm count, relative testes weight, seminiferous tubule density, sperm velocity, relative oviduct length, COC area and number of cumulus cells per oocyte than the focal monogamous species when the phylogenetic relationship between species was considered (Table 4), or was not (Table 5, Figure 1).

When we compared male versus female traits across species within the PGLS framework, we found that both seminiferous tubule density and sperm velocity in males positively correlates with cumulus cells per oocyte and relative oviduct length in females (Table 6, Figure 2), and that several relationships show non-significant trends but are greater than the *p*-values <0.05 threshold (Table 6).

Across species, we found that the mean relative testes weight positively correlates with mean sperm count, mean seminiferous tubule density positively correlates with mean sperm velocity, and that several relationships show non-significant trends but are greater than the *p*-values <0.05 threshold across species in males (Table 7). In females, we found that the mean number of cumulus cells per oocyte positively correlates with mean COC area and mean relative oviduct length within that species, but that COC area is not correlated with relative oviduct length across species (Table 7).

Within species, when we examined the association between testes weight and other male reproductive traits, we found no

TABLE 2 Mean and SD (standard deviation) of reproductive traits for each species

	Monogamous *Peromyscus*			Promiscuous *Peromyscus*		
Trait	***californicus***	***eremicus***	***polionotus***	***maniculatus***	***leucopus***	***gossypinus***
Testes weight (mg)	81.62 (24.21)	124.09 (33.06)	56.05 (8.99)	139.46 (18.70)	224.79 (32.53)	444.67 (68.65)
Seminiferous tubule density (%)	93.00 (2.59)	93.64 (1.17)	93.42 (3.14)	96.47 (1.18)	95.25 (1.66)	95.00 (2.33)
Sperm velocity (VCL; μm/s)	116.21 (31.43)	88.57 (16.66)	116.55 (18.49)	159.58 (30.43)	121.72 (10.96)	115.70 (19.55)
Sperm count	6.75×10^6 (2.93×10^6)	1.68×10^7 (1.11×10^7)	1.17×10^7 (7.50×10^6)	2.31×10^7 (5.94×10^6)	3.30×10^7 (1.87×10^7)	7.81×10^7 (1.89×10^7)
Oviduct length (mm)	10.21 (1.42)	8.40 (1.45)	10.18 (1.19)	14.87 (0.84)	12.96 (1.59)	14.96 (1.91)
COC area (mm^2)	0.18 (9.42×10^{-2})	0.16 (8.47×10^{-2})	0.13 (4.40×10^{-2})	0.23 (6.13×10^{-2})	0.19 (6.11×10^{-2})	0.16 (6.32×10^{-2})
Cumulus cells per oocyte	8.52×10^4 (5.17×10^4)	6.60×10^4 (4.83×10^4)	6.10×10^4 (2.00×10^4)	1.86×10^5 (6.52×10^4)	1.13×10^5 (8.92×10^4)	1.31×10^5 (8.0×10^4)
Male age (days)	208.17 (84.8)	346.96 (174.43)	154.68 (58.35)	187.71 (163.59)	286.76 (238.43)	155.06 (63.80)
Female age (days)	467.00 (250.69)	216.23 (191.25)	439.19 (240.47)	426.91 (303.99)	566.07 (333.82)	236.82 (109.74)
Male mass (mg)	35 762 (6081)	21 610 (2076)	15 144 (2180)	19 658 (2291)	20 375 (3162)	26 160 (5756)
Female mass (mg)	41 416 (11137)	20 155 (2743)	15 465 (1805)	19 540 (2477)	18 559 (4056)	23 178 (5446)
Female length (mm)	102.66 (7.41)	84.04 (6.03)	77.72 (2.81)	86.67 (3.34)	85.22 (3.55)	90.80 (4.03)

TABLE 3 Results of linear models examining differences in reproductive traits in six *Peromyscus* species

Fixed effect	Species	Estimate	SE	*t*-value	*p*-value
Sperm count	*P. californicus*	9.04×10^{6}	3.77×10^{6}	2.401	**0.019**
	P. eremicus	2.02×10^{7}	4.34×10^{6}	4.652	**<0.001**
	P. polionotus	1.32×10^{7}	3.46×10^{6}	3.821	**<0.001**
	P. maniculatus	2.47×10^{7}	3.47×10^{6}	7.107	**<0.001**
	P. leucopus	3.66×10^{7}	4.44×10^{6}	8.239	**<0.001**
	P. gossypinus	7.98×10^{7}	3.55×10^{6}	22.508	**<0.001**
	Model includes 'age' as a covariate				
Relative testes weight	*P. californicus*	3.86×10^{-3}	7.25×10^{-4}	5.329	**<0.001**
	P. eremicus	1.03×10^{-2}	9.34×10^{-4}	10.972	**<0.001**
	P. polionotus	6.31×10^{-3}	7.27×10^{-4}	8.678	**<0.001**
	P. maniculatus	1.36×10^{-2}	7.14×10^{-4}	19.075	**<0.001**
	P. leucopus	2.22×10^{-2}	7.37×10^{-4}	30.114	**<0.001**
	P. gossypinus	3.57×10^{-2}	7.03×10^{-4}	50.755	**<0.001**
	Model includes 'age' as a covariate				
Seminiferous tubule density	*P. californicus*	96.79	2.58	37.572	**<0.001**
	P. eremicus	95.30	1.52	62.903	**<0.001**
	P. polionotus	94.71	1.11	85.479	**<0.001**
	P. maniculatus	98.34	1.38	71.287	**<0.001**
	P. leucopus	96.75	1.44	67.084	**<0.001**
	P. gossypinus	97.22	1.59	61.180	**<0.001**
	Model includes 'age' and 'body weight' as covariates				
Sperm velocity	*P. californicus*	1.34×10^{2}	22.41	5.961	**<0.001**
	P. eremicus	1.03×10^{2}	15.93	6.433	**<0.001**
	P. polionotus	1.18×10^{2}	12.05	9.759	**<0.001**
	P. maniculatus	1.72×10^{2}	13.77	12.457	**<0.001**
	P. leucopus	1.50×10^{2}	15.05	9.972	**<0.001**
	P. gossypinus	1.41×10^{2}	19.66	7.183	**<0.001**
	Model includes 'age' and 'body weight' as covariates				
Cumulus cells per oocyte	*P. californicus*	6.51×10^{4}	5.87×10^{4}	1.109	0.271
	P. eremicus	5.60×10^{4}	3.25×10^{4}	1.724	0.089
	P. polionotus	5.27×10^{4}	2.85×10^{4}	1.848	0.068
	P. maniculatus	1.76×10^{5}	3.33×10^{4}	5.286	**<0.001**
	P. leucopus	1.02×10^{5}	3.32×10^{4}	3.316	**0.003**
	P. gossypinus	1.19×10^{5}	1.19×10^{4}	3.211	**0.002**
	Model includes 'body weight' as a covariate				
Relative oviduct length	*P. californicus*	0.199	9.86×10^{-3}	20.158	**<0.001**
	P. eremicus	0.201	8.04×10^{-3}	25.020	**<0.001**
	P. polionotus	0.264	1.03×10^{-3}	25.643	**<0.001**
	P. maniculatus	0.344	9.97×10^{-3}	34.509	**<0.001**
	P. leucopus	0.308	9.79×10^{-3}	31.494	**<0.001**
	P. gossypinus	0.326	8.46×10^{-3}	38.568	**<0.001**
	Model includes 'age' as a covariate				
COC area	*P. californicus*	0.164	0.030	7.154	**<0.001**
	P. eremicus	0.153	0.019	7.890	**<0.001**
	P. polionotus	0.119	0.021	5.726	**<0.001**
	P. maniculatus	0.217	0.021	10.379	**<0.001**
	P. leucopus	0.165	0.027	6.159	**<0.001**
	P. gossypinus	0.153	0.020	7.592	**<0.001**
	Model includes 'age' as a covariate				

Note: For each model, all rows were compared with an intercept of zero, the last row lists the covariates that yielded the best fitting model. *p*-Values < 0.05 are bolded.

TABLE 4 Results from phylogenetic generalized least-squares regression models explaining predictors of reproductive traits

Response	Predictor	*t*-value	*p*-value	Adjusted R^2	λ
Sperm count	Mating system	11.324	**0.007**	0.975	0 (1, 0.053)
	Sexual experience	2.104	0.170		
	Age	−2.798	0.108		
Relative testes weight	Mating system	33.151	**<0.001**	0.997	0 (1, 0.036)
	Sexual experience	8.355	0.241		
	Age	−4.867	0.354		
Seminiferous tubule density	Mating system	0.410	**<0.001**	0.237	0 (1, 0.003)
	Sexual experience	−1.101	0.351		
Sperm velocity	Mating system	−0.630	**0.006**	0.260	0 (1, 0.004)
	Sexual experience	−1.787	0.172		
COC area	Mating system	0.190	0.086	0.102	0 (1, 0.004)
	Body weight	0.565	0.612		
Cumulus cells per oocyte	Mating system	0.963	**0.030**	0.240	0 (1, 0.026)
	Body weight	0.231	0.832		
Relative oviduct length	Mating system	1.674	**0.005**	0.265	0 (0.279, 0.267)

Note: In all models, the branch length transformations for lambda, λ, were set using maximum likelihood ('ML'), with lower and upper boundaries for the lambda estimation indicated within parentheses. $p < 0.05$ are bolded.

significant correlations with sperm count, sperm velocity or seminiferous tubule density in any species (Figure S1), except a positive correlation between testes weight and sperm count in *P. gossypinus* ($r = 0.475$, $p = 0.046$).

4 | DISCUSSION

In this study, we investigated how mating strategy shapes the evolution of reproductive traits by quantifying trait variation within and between species of *Peromyscus* mice that have evolved under divergent mating systems. Like most rodents (Wolff, 2007), the majority of *Peromyscus* species are highly promiscuous meaning that both males and females mate with multiple partners during each reproductive cycle, but monogamy has evolved at least twice in the genus (reviewed in Bedford & Hoekstra, 2015; Turner et al., 2010). By focusing on species with established captive colonies, we reduced variance in our dataset due to environmental and social conditions, to examine how post-copulatory sexual selection has shaped the evolution of male and female reproductive traits. Our results support our prediction that males of the promiscuous species would display traits associated with increased investment in sperm production and swimming performance, and that females would exhibit traits that limit sperm access to ova, compared to the monogamous species. In addition, we found a strong positive relationship between traits associated with sperm competition and putative mechanisms of female control, which is consistent with coevolution of male and female traits driven by post-copulatory sexual selection.

Relative testes size is considered a robust indicator of sperm competition risk and is often used as a proxy for mating system when behavioural or genetic data are unavailable (Lüpold et al., 2020; Simmons & Fitzpatrick, 2012). Our results support these broadly observed trends and are consistent with evidence from wild caught *Peromyscus* (Linzey & Layne, 1969), showing that promiscuous species exhibit relatively larger testes than their monogamous congeners. A similar comparative study across the *Murinae* subfamily, which includes many mouse and rat species but not *Peromyscus*, found that populations with high intermale sperm competition exhibit average testes weight that is greater than one percent of their total body weight (Peirce & Breed, 2018). In our study, we found that all promiscuous species (*P. maniculatus*, *P. leucopus* and *P. gossypinus*) were above this one percent threshold, therefore, although these strains were reared under enforced monogamy in captivity (Bedford & Hoekstra, 2015), they still show a signature of intense sperm competition. In contrast, *P. polionotus* and *P. californicus* males fell below the one percent threshold, as expected of monogamous species (Peirce & Breed, 2018), yet *P. eremicus* fell slightly above. Interestingly, monogamy in *P. eremicus* is predicted to be 'facultative' and a consequence of mate scarcity rather than behavioural preference (Eisenberg, 1963). This prediction is consistent with our data, which indicates a greater investment in sperm production than expected from a truly monogamous species. While monogamy, in the strictest sense, has been predicted to have evolved prior to the divergence of *P. californicus* and *P. eremicus* (Turner et al., 2010), our findings suggest that *P. eremicus* may have retained aspects of their ancestral mating system as the two species diverged.

Testes size is largely driven by the amount of sperm-producing tissue, the seminiferous tubules, and is therefore expected to influence the number of sperm produced (reviewed in Simmons & Fitzpatrick, 2012). Across *Peromyscus* species, we found a strong positive correlation between testes size and sperm count, whether or not we scaled for differences in body size, indicating

TABLE 5 Results of best fitting linear mixed-effects models explaining predictors of reproductive traits

Sperm count	Fixed effects	Estimate	SE	*t*-value	*p*-value
	Intercept	-3.37×10^{7}	-8.70×10^{6}	−3.25	**0.002**
	Mating system	3.35×10^{7}	4.38×10^{6}	7.66	**<0.001**
	Age	-1.77×10^{4}	1.28×10^{2}	−1.38	**0.173**
	Body weight	6.83×10^{2}	3.03×10^{2}	2.25	0.274
	Random effect	SD			
	Species	5.83×10^{6}			
	Residual	1.23×10^{7}			
Relative testes weight	Fixed effects	Estimate	SE	*t*-value	*p*-value
	Intercept	1.47×10^{-2}	4.33×10^{-3}	3.39	**0.011**
	Mating system	1.87×10^{-2}	1.62×10^{-3}	11.55	**<0.001**
	Age	2.59×10^{-6}	2.59×10^{-6}	0.21	0.832
	Sexual experience	5.51×10^{-3}	5.51×10^{-3}	2.31	0.230
	Random effect	SD			
	Species	3.74×10^{-3}			
	Residual	1.96×10^{-3}			
Seminiferous tubule density	Fixed effects	Estimate	SE	*t*-value	*p*-value
	Intercept	95.25	0.329	289.6	**<0.001**
	Mating system	2.07	0.471	4.39	**<0.001**
	Age	2.09×10^{-4}	1.66×10^{-3}	0.13	0.900
	Body weight	-3.12×10^{-5}	2.63×10^{-5}	−1.41	0.161
	Random effect	SD			
	Species	2.26			
	Residual	2.09			
Sperm velocity	Fixed effects	Estimate	SE	*t*-value	*p*-value
	Intercept	89.42	7.02	12.74	**<0.001**
	Mating system	36.47	5.33	6.84	**<0.001**
	Sexual experience	−19.08	−19.08	8.05	−2.37
	Random effect	SD			
	Species	37.26			
	Residual	21.82			
COC area	Fixed effects	Estimate	SE	*t*-value	*p*-value
	Intercept	0.166	1.40×10^{-2}	11.89	**<0.001**
	Mating system	3.60×10^{-2}	1.53×10^{-2}	2.36	**0.021**
	Age	4.34×10^{-5}	3.00×10^{-5}	1.45	0.152
	Random effect	SD			
	Species	0.019			
	Residual	0.070			
Cumulus cells per oocyte	Fixed effects	Estimate	SE	*t*-value	*p*-value
	Intercept	7.73×10^{4}	1.23×10^{-4}	6.28	**0.002**
	Mating system	7.66×10^{4}	1.39×10^{-4}	5.52	**<0.001**
	Age	−52.99	26.91	−1.97	0.052
	Random effect	SD			
	Species	5.10×10^{4}			
	Residual	6.16×10^{4}			

TABLE 5 (Continued)

Relative oviduct length	Fixed effects	Estimate	SE	*t*-value	*p*-value
	Intercept	0.239	2.24×10^{-2}	10.66	**<0.001**
	Mating system	0.10	7.89×10^{-3}	13.28	**<0.001**
	Random effect	SD			
	Species	0.043			
	Residual	0.030			

Note: *p*-Values < 0.05 are bolded.

that species with larger testes produce more sperm. This pattern has been observed in a number of interspecific comparative studies (e.g. in fish [Stockley et al., 1997], reptiles [Friesen et al., 2017], birds [Garamszegi et al., 2005], mammals [Cordeiro et al., 2021; Møller, 1989] and insects [Hayward & Gillooly, 2011]), however, it contrasted with our intraspecific results in which only one of the six focal species, *P. gossypinus*, showed a correlation between testes size and sperm count across males. Notably, we found that variation in both of these traits is greater across than within species, which can make correlations more likely to be detected (Mincey, 2018) and, thus, may account for the differences between our inter- and intraspecific analyses. Moreover, differences in sperm count can also be regulated by sperm production kinetics and testicular architecture (Roldan, 2019), such as seminiferous tubule density (De León-Ramírez et al., 2021; Firman et al., 2018; França & Godinho, 2003). In our study, the promiscuous species exhibited a higher density of seminiferous tubules than the monogamous species, but we did not observe a significant correlation between seminiferous tubule density and sperm count, which is consistent with an earlier finding in humans (van Dop et al., 1980). Together our results suggest that in *Peromyscus*, selection for increased sperm number is likely driving interspecific variation in testes size, rather than seminiferous tubule density. Surprisingly, we found a positive correlation between seminiferous tubule density and sperm velocity across species. Sertoli cells are the dominant cell type, aside from developing spermatozoa, within the seminiferous tubules and function to nurture and support developing sperm (França et al., 2016; Griswold, 2018). In promiscuous populations of red deer (*Cervus elaphus*), males exhibit larger Sertoli cells and more dense seminiferous tubules compared to monogamous populations (Pintus et al., 2015), which suggests a higher investment in support tissue in response to sperm competition. It is possible that improved sperm swimming performance in *Peromyscus* may be due to an increase in investment in spermatogenic tissue, especially since differences in sperm length among these species (Linzey & Layne, 1974) may be associated with differential development within the seminiferous tubules, but further study is required to understand how these two traits functionally relate.

In many taxa and especially mammals, polyspermy can cause aberrant embryo development and embryonic death (Hunter, 1996). The risk of polyspermy increases with the number of sperm within the female tract (Hunter, 1996) and near the oocyte (Shin et al., 2003). Cryptic female choice theory predicts that females evolve mechanisms to reduce risk to their investment, their ova (Eberhard, 1996), and in internally fertilizing species, the female reproductive tract efficiently reduces sperm access to oocytes. In most mammals, for example, hundreds of millions of sperm can be released in an ejaculate (Mahé et al., 2021), but only hundreds will make it to the oviduct (Suarez, 2008), and as few as a dozen sperm may ever reach the oocyte (Kölle, 2015). Adaptations that further reduce the number of sperm at the fertilization site are expected to reduce polyspermy and are especially important if males are capable of ejaculating large numbers of sperm (Eberhard, 1996; Firman et al., 2017). We found that in *Peromyscus*, males of promiscuous species produce significantly more sperm, and accordingly, female conspecifics exhibited longer oviducts, which is predicted to restrict sperm access to the ampulla in the distal end of the oviduct, and a larger cumulus cell mass (i.e. area and cell count), which is further expected to limit sperm access to oocytes, compared to monogamous species. While cumulus cell number and area are rarely studied from an evolutionary perspective, our oviduct length results are consistent with other comparative studies (Anderson et al., 2006; Gomendio & Roldan, 1993) showing that length is positively associated with polyandry. Evidence supporting the importance of oviduct length and cumulus cells in limiting sperm access can be observed in mammalian *in vitro* fertilization studies, which can achieve fertilization by less motile sperm by removing the need for sperm migration and by denaturing cumulus cells with hyalaronidase to enable sperm to gain access to the oocyte (Buderatska et al., 2020). These *in vitro* manipulations, however, lead to a high degree of polyspermy, which can be two to three times more frequent than observed in natural matings (Mahé et al., 2021; Shin et al., 2003), further supporting their role in cryptic female choice. In addition to this adaptive explanation for the increased cumulus number in promiscuous species, since cumulus cells and Sertoli cells share similar developmental origins from the coelomic epithelium (Karl & Capel, 1998; Pereda et al., 2006), it is possible that genetic and hormonal pathways that yield greater Sertoli cell numbers in males of species with intense sperm competition, may also increase cumulus cell number in the females of such species. Testing these predictions, and the functional relevance of oviduct length and cumulus cells in preventing polyspermy, are important next steps to understand how post-copulatory sexual selection might act on female traits in *Peromyscus*.

Reducing the occurrence of polyspermy protects female investment in ova, but females may also bias fertilization of their oocytes by selecting specific sperm or a subpopulation of the male's ejaculate (Holt & Fazeli, 2016) to improve their fitness (Eberhard, 1996).

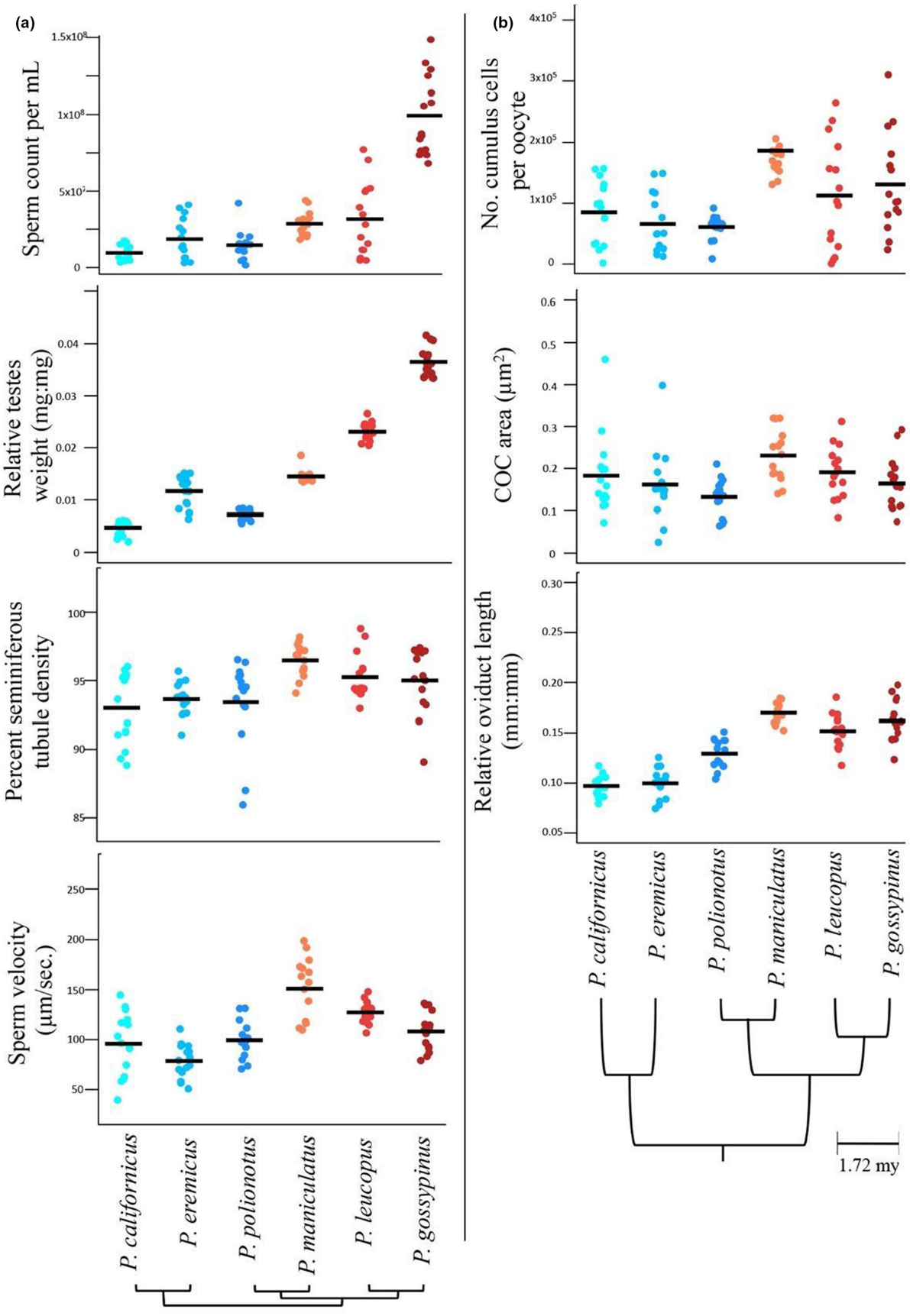

FIGURE 1 Reproductive trait variation within and between *Peromyscus* species. Dots represents individual (a) male and (b) female trait values. Black bars indicate species mean. Monogamous species are shown as cool colours (teal, light-blue and blue) and promiscuous species are shown as warm colours (orange, red and crimson). Species relatedness is shown below each *x*-axis.

TABLE 6 Results from phylogenetic generalized least squares correlations explaining intersexual trait associations across species

Male trait	Female trait	Estimate	SE	*r*	*p*-value
Sperm count	COC area	2.27×10^{-9}	2.40×10^{-9}	−0.022	0.398
Sperm count	Cumulus cells per oocyte	3.66×10^{-3}	2.86×10^{-3}	0.114	0.269
Sperm count	Relative oviduct length	2.60×10^{-9}	1.78×10^{-9}	0.184	0.218
Relative testes weight	COC area	17.317	7.836	0.437	0.092
Relative testes weight	Cumulus cells per oocyte	2.42×10^{7}	8.79×10^{6}	0.573	0.360
Relative testes weight	Relative oviduct length	16.509	5.137	0.651	0.083
Seminiferous tubule density	COC area	0.032	1.64×10^{-3}	0.987	0.094
Seminiferous tubule density	Cumulus cells per oocyte	4.11×10^{4}	2.04×10^{3}	0.988	**<0.001**
Seminiferous tubule density	Relative oviduct length	0.020	2.06×10^{-3}	0.971	**<0.001**
Sperm velocity	COC area	1.88×10^{-3}	8.28×10^{-5}	0.990	0.064
Sperm velocity	Cumulus cells per oocyte	2.38×10^{3}	201	0.966	**<0.001**
Sperm velocity	Relative oviduct length	1.54×10^{-3}	1.71×10^{-4}	0.941	**<0.001**

Note: *p*-Values < 0.05 are bolded.

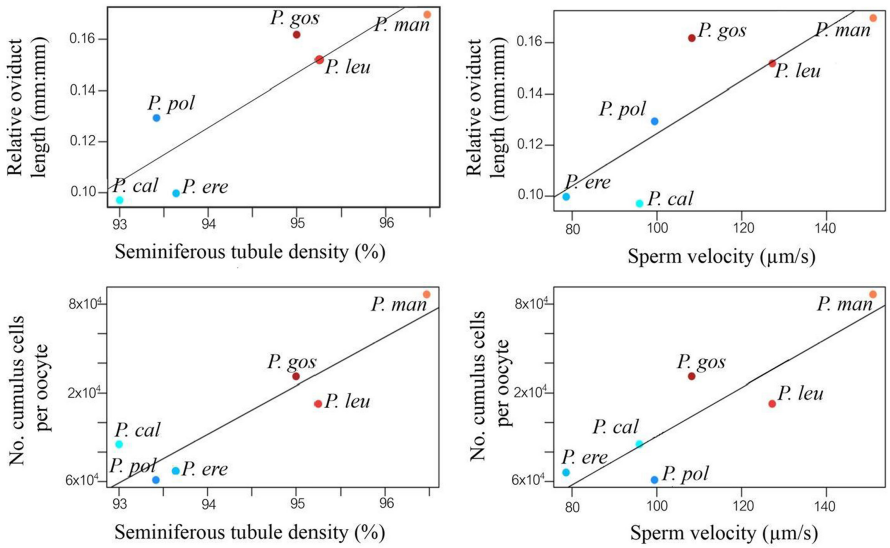

FIGURE 2 Statistically significantly intersexual correlations comparing reproductive traits across three monogamous species, *P. californicus* (*P. cal*), *P. eremicus* (*P. ere*) and *P. polionotus* (*P. pol*), and three promiscuous species, *P. gossypinus* (*P. gos*), *P. leucopus* (*P. leu*) and *P. maniculatus* (*P. man*). Monogamous species are shown as cool colours (teal, light-blue and blue) and promiscuous species are shown as warm colours (orange, red and crimson).

TABLE 7 Results from phylogenetic generalized least squares correlations explaining intrasexual trait associations across species

Trait I	Trait II	Estimate	SE	*r*	*p*-value
Within males					
Sperm count	Relative testes weight	3.99×10^{9}	9.32×10^{8}	0.776	**0.013**
Sperm count	Seminiferous tubule density	2.98×10^{6}	2.65×10^{6}	0.051	0.323
Sperm count	Sperm velocity	1.16×10^{-6}	1.28×10^{-6}	−0.035	0.414
Relative testes weight	Seminiferous tubule density	109×10^{-3}	4.23×10^{-4}	0.531	0.061
Relative testes weight	Sperm velocity	9.04×10^{3}	4.21×10^{3}	0.420	0.098
Seminiferous tubule density	Sperm velocity	16.961	0.948	0.985	**<0.001**
Within females					
COC area	Cumulus cells per oocyte	1.27×10^{9}	9.13×10^{9}	0.975	**<0.001**
COC area	Relative oviduct length	0.080	0.100	0.565	0.243
Cumulus cells per oocyte	Relative oviduct length	6.45×10^{-7}	4.54×10^{-8}	0.976	**<0.001**

Note: *p*-Values < 0.05 are bolded.

Mechanisms that reduce sperm number in the ampulla, such as increased oviduct length, may also select for sperm cells with greater longevity or velocity (Holt & Fazeli, 2010; Rickard et al., 2019). Across *Peromyscus*, we found a strong positive correlation between sperm velocity (and seminiferous tubule density) in males and oviduct length in females, which is consistent with coevolution among the sexes in other systems (reviewed in Birkhead & Montgomerie, 2020; Holt & Fazeli, 2010). Additionally, we found that sperm swimming velocity (and seminiferous tubule density) is also positively correlated with the number of cumulus cells surrounding each oocyte. Cumulus cells produce and secrete progesterone (Schuetz & Dubin, 1984), which has been shown to attract sperm in some species (Guidobaldi et al., 2008; but see Suarez, 2008). Therefore, even though a greater number of cumulus cells may produce more chemoattractant, they also pose a greater physical barrier to fertilization. While it remains unclear how specific male traits interact with cumulus cell density or oviduct length in *Peromyscus*, our data indicate that traits associated with sperm velocity, rather than increased sperm number, are most likely to be targets of selection in this system.

Together, our results support the prediction that male and female traits evolve in a coordinated fashion, driven simultaneously by post-copulatory sexual selection and divergent optima between the sexes (Arnqvist & Rowe, 2005), and add to a growing body of empirical examples (e.g. fruit flies [*Drosophila*; Miller & Pitnick, 2002], bean weevil [*Coleoptera bruchidae*; Rönn et al., 2007], waterfowl [Brennan et al., 2007] and land snails [*Arianta arbustorum*; Beese & Baur, 2006]). Perhaps unsurprisingly, we find that not all species within the *Peromyscus* lineage respond to post-copulatory sexual selection similarly; for example, *P. maniculatus* invest more in sperm swimming performance (i.e. sperm 'quality'), whereas *P. gossypinus* males invest more in sperm number (i.e. sperm 'quantity') and the intraspecific data indicate this is largely driven by increased testes size. Moreover, we find that the proposed mechanisms of female control, oviduct length and number of cumulus cells correlate with sperm quality traits (velocity and seminiferous tubule density), rather than testes size and sperm count, similar to evidence of co-evolving female reproductive tract fluids with sperm velocity in other species (reviewed in Gasparini et al., 2020). Like much work on post-copulatory sexual selection (Orr et al., 2020), our understanding of mechanisms of female control in *Peromyscus* lags behind our understanding of sperm and ejaculate competition. Here we propose rarely considered traits, number of cumulus cells per oocyte and COC area, as important adaptations to post-copulatory sexual selection in mammals. By studying male and female traits in tandem, we not only identify possible targets of selection, but we also develop predictions regarding the functional significance of these traits.

AUTHOR CONTRIBUTIONS

William David Weber: Data curation (lead); formal analysis (lead); investigation (lead); methodology (supporting); project administration (supporting); validation (lead); visualization (lead); writing – original draft (equal); writing – review and editing (equal). **Heidi S Fisher:** Conceptualization (lead); data curation (supporting); formal analysis (supporting); funding acquisition (lead); investigation (supporting); methodology (supporting); project administration (lead); resources (lead); supervision (lead); validation (supporting); visualization (supporting); writing – original draft (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data, R-programing script, and a README file used to run the statistical analysis that support the findings of this study are openly available in Dryad at https://datadryad.org/stash/dataset/doi:10.5061/dryad.2fqz612rm. All raw image files used for analysis, and five representative live sperm videos for each focal species are available for download at https://umd.box.com/v/WeberFisher2022a. While under review, referees can access this material with the following link; https://datadryad.org/stash/share/pvTSHb-RD-Mzfkq5jj8KMa4wXDmEyrvA6nZEJ_e24Un0

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