



# Indirect genetic effects and sexual conflicts: Partner genotype influences multiple morphological and behavioral reproductive traits in a flatworm

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The expression of an individual's phenotypic traits can be influenced by genes expressed in its social partners. Theoretical models predict that such indirect genetic effects (IGEs) on reproductive traits should play an important role in determining the evolutionary outcome of sexual conflict. However, empirical tests of (i) whether reproductive IGEs exist, (ii) how they vary among genotypes, and (iii) whether they are uniform for different types of reproductive traits are largely lacking. We addressed this in a series of experiments in the simultaneously hermaphroditic flatworm *Macrostomum lignano*. We found strong evidence for IGEs on both morphological and behavioral reproductive traits. Partner genotype had a significant impact on the testis size of focal individuals—varying up to 2.4-fold—suggesting that IGEs could mediate sexual conflicts that target the male sex function. We also found that time to first copulation was affected by a genotype × genotype interaction between mating partners, and that partner genotype affected the propensity to copulate and perform the postcopulatory suck behavior, which may mediate conflicts over the fate of received ejaculate components. These findings provide clear empirical evidence for IGEs on multiple behavioral and morphological reproductive traits, which suggests that the evolutionary dynamics of these traits could be altered by genes contained in the social environment.

**KEY WORDS:** G × G interaction, interacting phenotype, sexual conflicts, social effects, sperm competition, testis evolution.

The concept of extended phenotypes (Dawkins 1982) proposes that an individual's genes can have phenotypic effects “outside” of the carrier's body. Such extended phenotypes might be expected to affect evolutionary dynamics, in that evolutionary responses do not only depend on the genes expressed in the individual that expresses the phenotype, but also on the genes contained in its environment (Bailey 2012). In particular, an individual's phenotype could potentially be altered by genes expressed by conspecific individuals that it interacts with (indirect genetic effect, IGE, Moore et al. 1997; Wolf et al. 1998; Wolf 2003). IGEs have

particular evolutionary importance for behaviors expressed during social interactions (Moore et al. 1997; Wolf et al. 1998, 2014; Bleakley et al. 2010; Schneider et al. 2016), such as aggressive behavior (Saltz 2013; Camerlink et al. 2015), mate choice (Chenoweth et al. 2010; Bailey and Moore 2012; Billeter et al. 2012) or sexual conflict (Moore and Pizzari 2005; González-Forero and Gavrillets 2013).

Specifically, sexual conflict over courtship, mating, and its outcomes is a context in which IGEs are expected to play a particularly significant role (Moore and Pizzari 2005). For instance,

in *Drosophila melanogaster*, copulation duration (i.e., a trait subject to sexual conflict) is determined by both female and male genetic effects (Edward et al. 2014). However, apart from this latter study, there have been very few controlled empirical studies testing the potential for IGEs to impact on sexual conflict. This is despite theoretical predictions that (i) relatively flexible and plastic behavioral and morphological traits that shape courtship and mating should be more easily modified by social partners, and thus more susceptible to IGEs, (ii) multiple reproductive traits could potentially respond to partner genotypes, and (iii) IGEs on traits that mediate sexual conflict will likely depend on both partners' genotypes, as a result of genotype-by-genotype interactions ( $G \times G$ ) (Moore and Pizzari 2005). Such  $G \times G$  interactions result from the joint action of male persistence adaptations, which, for example, could alter the physiology or behavior of the sperm recipient, and female resistance adaptations, which counteract and/or mediate the effects of the male persistence adaptations (Moore and Pizzari 2005; González-Forero and Gavrillets 2013). This in turn can lead to sexually antagonistic coevolution that continuously favors the evolution of novel male persistence and female resistance traits (Arnqvist and Rowe 2005), and may thus impact multiple male and female traits (Moore and Pizzari 2005). Here, we empirically test these theoretical predictions by investigating the outcomes of specific genotypic interactions among partners, to examine whether IGEs on reproductive behavior and morphology could create the opportunity for sexual conflict.

Even though sexual conflict has primarily been studied in gonochorists (i.e., species with separate sexes), it clearly also occurs in simultaneous hermaphrodites, which, because they combine male and female sex functions in a single individual, may experience and respond to sexual conflicts differently (Charnov 1979; Michiels 1998; Arnqvist and Rowe 2005; Schärer 2009; Schärer and Janicke 2009; Schärer et al. 2014). For instance, conflict may arise over the mating role. A simultaneous hermaphrodite may benefit from either donating and/or receiving sperm, that is acting in the male and/or female mating role. This leads to a mating conflict if the partners do not have compatible mating interests, for example, when both would benefit more from donating rather than receiving sperm (Michiels 1998; Schärer et al. 2014). Such a conflict can be resolved if the partners agree to assume both mating roles (i.e., donate and receive sperm), leading either to reciprocal copulations or to unilateral copulations with conditional reciprocity—two common modes of copulation across simultaneous hermaphrodites (Michiels 1998; Schärer et al. 2014). However, such conflict resolution at the point of copulation might lead to a shift of the conflict to the postmating arena, where sperm donors and recipients may then have divergent interests over the fate of the transferred ejaculate (Charnov 1979; Schärer et al. 2011, 2014).

Several lines of evidence with respect to reproductive trait evolution in the free-living flatworm genus *Macrostomum* can be seen in the light of such sexual conflicts. Specifically, reciprocally mating species of the genus exhibit an intriguing postcopulatory suck behavior, during which the sperm recipient places its pharynx over its own female genital opening, presumably attempting to remove ejaculate components out of the sperm storage organ after mating. The suck behavior appears to represent a female resistance trait, whereas the corresponding male persistence trait is a sperm morphology with stiff lateral bristles, thought to be involved in preventing the removal of sperm during suck behavior (Schärer et al. 2004, 2011; Vizoso et al. 2010; Marie-Orleach et al. 2013).

In addition to postmating conflicts—which can often be conceptually similar to those observed between males and females in gonochorists (Chapman 2001; Arnqvist and Rowe 2005; Sirot et al. 2015)—simultaneous hermaphrodites may also experience conflict over the optimal sex allocation of the partner, a conflict that is rather unique. This is because a sperm donor gains fitness primarily via the female sex function of the partner, whereas the male sex function of that partner may often not be beneficial to the sperm donor, at least once the sperm donor has received sufficient sperm to fertilise its own eggs (Charnov 1979; Schärer 2009, 2014; Schärer and Janicke 2009; Nakadera et al. 2014; Schärer et al. 2014). There are several reasons for this. First, assuming a trade-off between allocation to the male and female function (Schärer et al. 2005; Schärer 2009), all the resources the partner allocates to its male sex function are not channelled into the production of eggs, which could have been fertilized by the sperm donor. Second, the partner might be a future sperm competitor of the sperm donor, and so may increase the risk and intensity of sperm competition experienced by the sperm donor in future mating opportunities. Third, the partner might eventually mate (again) in the male role with the former sperm donor and at that point transfer potentially unwanted and/or harmful ejaculate. For these reasons, the male sex function of the partner represents a prime target of manipulation for sperm donors (Schärer 2009, 2014; Nakadera et al. 2014; Schärer et al. 2014). In support of this, Nakadera et al. (2014) have shown that in the great pond snail, *Lymnaea stagnalis*, the sperm donor transfers a seminal fluid protein during mating, which upon receipt causes a striking reduction in the amount of sperm transferred and paternity success obtained in subsequent inseminations by the sperm recipient—though it is currently still unclear if that actually increases the sperm donor's fitness (Schärer 2014).

If there are sexual conflicts over the outcome of mating and sex allocation, then we can expect the underlying persistence and resistance traits to evolve under sexually antagonistic coevolution (Arnqvist and Rowe 2005; González-Forero and Gavrillets 2013). Populations may thus exhibit genetic polymorphisms in

persistence and resistance alleles, where, for instance, certain alleles are effective in manipulating certain partners but fail to manipulate others (to the same degree). Thus for ongoing sexually antagonistic coevolution to be possible in a population, it is necessary for some reproductive traits of an individual to be predictably affected by the genotypic variation among its potential mating partners, or in other words, for certain reproductive traits to exhibit IGEs. This prerequisite can be experimentally tested by crossing different genotypes, and measuring IGEs on putative morphological and behavioral targets of sexual conflict. In other words, identifying such IGEs represents a possible first step toward showing that some traits have the potential to be targets for sexual conflict (though documenting whether they actually evolve due to sexually antagonistic coevolution, as opposed to, for example, a mutually beneficial interaction, then represents an important second step).

In a series of three separate experiments, we empirically tested the effects of the partner's genotype on a focal individual's expression of a range of morphological and behavioral reproductive traits that we expected to mediate sexual conflicts. Specifically, we crossed inbred lines of the simultaneously hermaphroditic free-living flatworm, *Macrostomum lignano*, in different experiments ideally suited to study specific questions and traits. In a first full factorial ( $4 \times 4$ ) experiment, we measured a suite of morphological traits expressed by focal individuals (stemming from one of four lines) that had grown up together with a partner worm (stemming from one of four other lines). This design allowed us to partition the sources of morphological variation to the focal line, the partner line, and their interaction. The measured morphological traits included body, testis, ovary, and seminal vesicle size. IGEs affected the male sex function, as both testis and seminal vesicle size were contingent on the genotype of the partner, so we also estimated the interaction effect coefficient  $\psi$  (Moore et al. 1997) for all focal traits. Quantifying  $\psi$  allowed us to identify putative partner traits underlying phenotypic responses in focal individuals. The magnitude of  $\psi$  describes how much the expression of a focal trait depends on a partner trait, and its sign describes whether the focal trait value increases or decreases with the partner trait (Moore et al. 1997; Bleakley et al. 2010; Bailey and Hoskins 2014).

In a second one-way ( $1 \times 7$ ) experiment, we measured morphological traits and copulation rates of focal individuals derived from one single line, which were paired with individuals derived from seven different lines, a design that allowed us to use more partner lines and a better replication per partner line (helping to detect rarer line effects and smaller effect sizes). The results corroborated the finding that IGEs affected testis and seminal vesicle size, and also suggested that copulatory interactions could depend on the partner line.

Finally, in a third full-factorial ( $4 \times 4$ ) experiment, we quantified several copulatory behaviors—including time to first copulation, copulation duration, and copulation termination—as well as the postcopulatory suck behavior expressed by both paired individuals. In this experiment, the worms were virgins when they were first paired for observation, while in the other experiments they had grown up together and thus mated many times before. Both time to first copulation and the number of sucks were influenced by partner genotype. Taken together, our results illustrate how IGEs and  $G \times G$  interactions shape the expression of morphological and behavioral reproductive traits, and we discuss the implications for the evolution of traits that mediate sexual conflicts.

## Materials and Methods

### STUDY ORGANISM

*Macrostomum lignano* (Macrostomorpha, Platyhelminthes) is a free-living flatworm species of the intertidal meiofauna of the Northern Adriatic Sea (Ladurner et al. 2005b). Relatively little is currently known about their ecology and mating system under natural conditions, but we know that they occur in very variable densities, often have received sperm when freshly collected in the field, and look very similar in overall morphology, though are often smaller in size (Schärer, pers. obs.). Under laboratory conditions, worms are cultured at 20°C in glass Petri dishes filled with *f/2* medium (Andersen et al. 2005) and fed with the diatom *Nitzschia curvilineata*. This simultaneously hermaphroditic worm is highly transparent, which allows us to measure many morphological traits noninvasively, including body, testis, ovary, and seminal vesicle size (Schärer and Ladurner 2003). Individuals with larger testes produce more sperm, successfully transfer more sperm per copulation, and thus sire more offspring (Schärer and Vizoso 2007; Schärer and Janicke 2009; Marie-Orleach et al. 2016). Moreover, individuals show phenotypic plasticity in response to group size: in large groups individuals grow bigger testes and smaller ovaries than in small groups, which is argued to be an adaptive sex allocation adjustment in response to the current level of sperm competition (Schärer and Ladurner 2003; Schärer et al. 2005; Janicke et al. 2013). In the laboratory, *M. lignano* acquires sexually maturity in both sex functions  $\sim 13$  days after hatching (Schärer and Ladurner 2003), and is then promiscuous (Schärer et al. 2004; Janicke and Schärer 2009) and copulates frequently (in pairs it on average mates about six times per hour; Schärer et al. 2004; Ladurner et al. 2005b). This high mating rate is somewhat surprising considering that the worms only lay about one egg per day (Janicke et al. 2011), and may indicate that mating may be primarily motivated by sperm donation rather than sperm receipt (Vizoso et al. 2010). Copulation consists of the reciprocal insertion of the male copulatory organ into the female genital organ

of the partner for ~10 s. Copulation is often followed by a striking postcopulatory suck behavior, in which individuals place their pharynx over their own female genital opening and appear to suck out ejaculate components (for details about the mating behavior, see Schärer et al. 2004; Vizoso et al. 2010). This behavior is performed after about 50% of copulations (Schärer et al. 2004), and its occurrence has recently been shown to depend on the mating status of the sperm donor (i.e., sperm recipients suck less often after copulating with a virgin individual compared to a sexually experienced individual; Marie-Orleach et al. 2013). Because virgin individuals carry greater amounts of (accumulated) prostate gland secretions (and are thus presumably capable of transferring greater amounts of secretions per mating), this effect has been interpreted as being mediated by the prostate gland secretions transferred during mating, which can presumably prevent the sperm recipient from sucking (Marie-Orleach et al. 2013).

### GFP(+) AND GFP(-) GENETIC LINES

To keep track of individuals within pairs, we made use of individuals from genetic lines that either do or do not express green fluorescent protein (GFP), and these GFP(+) and GFP(-) genetic lines were both expected to show high levels of homozygosity. The GFP(-) lines, called “DV lines,” were generated through several generations of full- and half-sib inbreeding. Details about the breeding design used to establish the DV lines are described elsewhere (Janicke et al. 2013; Vellnow et al., in prep).

The GFP(+) genetic lines, called “LM lines,” were established with the help of the transgenic HUB1 line (Demircan 2013; Marie-Orleach et al. 2014), which shows ubiquitous expression of GFP. Fitness assays indicate that HUB1 individuals do not differ significantly from wild-type individuals in their morphology, mating rates, or reproductive success (Marie-Orleach et al. 2014). The LM lines were generated by backcrossing HUB1 worms onto several DV lines. Specifically, we paired HUB1 with juvenile DV individuals (four replicates per line), and used the resulting GFP(+) offspring to pair with another juvenile DV worm of the corresponding line. We repeated this procedure over eight generations, producing individuals genetically almost identical to the corresponding DV lines (Hartl and Clark 1997), but heterozygous for the GFP locus. Third, to create individuals homozygous for the GFP locus, we (i) pooled F8 GFP(+) individuals within each line, (ii) used the resulting GFP(+) offspring to pair with an individual of the corresponding DV line, and (iii) screened the GFP status of the offspring produced. When we observed 100% of GFP(+) offspring in at least 10 offspring, the GFP(+) individual was considered to be homozygous for the GFP locus. (iv) The latter individuals were then sampled and pooled to initiate the LM lines. Note that we discarded the offspring produced during the first 14 days (to avoid egg fertilization by sperm of the previous DV partners). The LM lines were then maintained at a popula-

tion size of 10 individuals. We started with 37 LM lines but, due to a lack of offspring produced in either the initial backcrossing ( $n = 8$ ) or in the subsequent establishment of homozygote LM lines ( $n = 9$ ), the final number of LM lines was reduced to 20, 17 of which were used here.

### EXPERIMENT 1: REPRODUCTIVE MORPHOLOGY

To test for the presence and magnitude of direct and indirect genetic effects on reproductive morphology, we assessed a number of traits in individuals raised in pairs, each consisting of one GFP(-) worm from one of four inbred DV lines and one GFP(+) worm from one of four inbred LM lines, in all possible 16 combinations for a  $4 \times 4$  full factorial design. To minimize potential confounding effects of the common rearing environment experienced by the worms within the mass cultures of their inbred lines, the F0 parents of all F1 worms used in the experiment were held under common conditions before the experiment. For this, we sampled subadult and adult F0 individuals from the mass cultures from two sets of four GFP(-) DV lines (set 1: DV10, DV25, DV37, and DV81; set 2: DV6, DV12, DV20, and DV33) and, for the set 2, we also sampled F0 individuals of the corresponding GFP(+) LM lines (LM6, LM12, LM20, and LM33). All these individuals were isolated for 3 weeks in a balanced manner in wells of 24-well plates (TPP AG, Switzerland) under ad libitum food conditions. This period of isolation was chosen to maximize the likelihood that worms had matured, and no longer stored any received sperm from earlier matings. We then formed pairs from F0 parental individuals within inbred lines that were either always GFP(-)  $\times$  GFP(-) (i.e., DV10  $\times$  DV10, DV25  $\times$  DV25, DV37  $\times$  DV37, and DV81  $\times$  DV81) or always GFP(-)  $\times$  GFP(+) (i.e., DV6  $\times$  LM6, DV12  $\times$  LM12, DV20  $\times$  LM20, and DV33  $\times$  LM33). These pairs were kept for 4 weeks, eventually yielding GFP(-) and GFP(+) F1 inbred individuals, respectively, that were then used as the experimental animals. Note that only half of the offspring produced by GFP(-)  $\times$  GFP(+) crosses were expected to be GFP(+) because GFP expression is dominant, and the GFP(+) parents were themselves expected to be heterozygotes due to the ongoing backcrossing (see Marie-Orleach et al. 2014 for details on inheritance patterns).

On day 1, we paired the F1 individuals, crossing GFP(-) with GFP(+) lines in a  $4 \times 4$  full factorial design, which allowed us to identify each individual within a pair based on the worms' GFP status. In all replicates, the GFP(+) individual was a priori designated as the focal, and the GFP(-) as the partner. The individuals were expected to become sexually active a few days after pair formation, and we transferred the pairs to fresh algae every two weeks to provide ad libitum food conditions and to limit social interactions between the adult individuals and their produced offspring. On day 62 and 66, we took digital micrographs of the GFP(+) focal individual of all pairs using a Leica DM2500 microscope

(Leica Microsystems, Germany), a digital camera (DFK 41AF02, The Imaging Source Europe GmbH, Germany) and the software BTV Pro 6.0b3 (<http://www.bensoftware.com/>), following the protocol described in Schärer and Ladurner (2003). We then analyzed the micrographs using ImageJ 1.39u (<http://rsb.info.nih.gov/ij/>), which allowed us to estimate body, testis, ovary, and seminal vesicle size. All these measures have previously been shown to have good repeatabilities (Schärer and Ladurner 2003).

From an initial 198 pairs, 30 were lost due to either incomplete development of the worms or handling errors during measurements. The final number of pairs was thus reduced to 168, that is on average 10.5 biological replicates per cross-combination (range: 6–15).

First, we tested whether the phenotypes of focal individuals are influenced by their respective genotypes, the genotypes of their partners, or both. For this, we tested the effects of the focal line, the partner line and the focal line  $\times$  partner line interaction on body, testis, ovary, and seminal vesicle size, using two-way ANOVAs. Testis and seminal vesicle size were log transformed, and ovary size was square root transformed to approach normality.

Second, we studied the relationships between morphological traits expressed by focal individuals and those expressed by their partners, by estimating the interaction effect coefficient  $\psi$  for each pairwise combination of traits (Moore et al. 1997). To do this, we separately modeled each of the four focal traits,  $z_i$ :

$$z_i = \alpha_i + \beta_i f + \sum_{j=1}^4 \psi_{ij} z'_j + \sum_{j=1}^4 \gamma_{ij} (f \times z'_j) + \varepsilon_i$$

Focal line identity,  $f$ , was a fixed factor with four levels representing the four focal genotypes tested, and coefficient  $\beta_i$ . Each partner trait  $z'_j$  (the prime indicates the trait is expressed in an interacting partner, and the traits modeled were: body, testis, ovary, and seminal vesicle size) is a continuous variable with coefficient  $\psi_{ij}$ . The  $\psi$  values thus describe the direction and magnitude of the association between focal trait expression and partner trait expression, and are equivalent to partial regression coefficients (as in eq. (3) in Moore et al. 1997). We also modelled each two-way interaction term between focal line and partner trait ( $f \times z'_j$ ) to specifically test whether  $\psi$  was similar across the four focal lines. Coefficients for the interaction terms are denoted  $\gamma_{ij}$ , and  $\alpha_i$  and  $\varepsilon_i$  denote the intercept and error, respectively. For each focal trait, the model produced four corresponding estimates of  $\psi$ , plus four corresponding interaction terms, so in total we produced a full complement of 16  $\psi$  estimates and 16 interaction terms testing for heterogeneity in  $\psi$  across focal lines. Prior to entry into the model, partner body size was square root transformed, and partner testis, ovary, and seminal vesicle size were log transformed. All focal and partner traits were standardized (i.e., mean = 0, SD = 1) to facilitate quantitative comparisons among traits, and

among other published studies that have estimated  $\psi$ . Note that due to handling errors when measuring partner individuals, the number of pairs used to estimate  $\psi$  was reduced by an additional 37 pairs to 131 pairs, that is on average 8.2 biological replicates per cross (range: 4–14).

## EXPERIMENT 2: REPRODUCTIVE MORPHOLOGY AND BEHAVIOR

Here, we tested for genetic effects on both morphological and behavioral traits by measuring individuals of one inbred line that were exposed to partners from one of seven different inbred lines, that is a  $1 \times 7$  one-way design (again controlling for common rearing environment). We first sampled 100 F0 individuals from eight lines (i.e., HUB1 as the focal GFP(+) line, and seven partner GFP(-) lines (namely DV8, DV12, DV29, DV35, DV71, DV81, and DV84) and placed them into eight separate Petri dishes where they were allowed to lay eggs. We then sampled the resulting F1 offspring and formed intra-line pairs in well plates. These pairs were regularly transferred to fresh algae for nine weeks, after which we formed intra-line triplets to facilitate offspring production. The resulting F2 inbred offspring were then used as our experimental animals.

On day 1, we sampled two F2 offspring in each triplet, and paired a focal individual of the GFP(+) line with a partner from one of the seven GFP(-) lines. These pairs were then regularly transferred to fresh algae. On days 55 and 59, we assessed the copulation rate of each pair. For this, we placed the pairs in 4  $\mu$ L drops of artificial sea water in observation chambers, as described in Schärer et al. (2004), including 12 drops per observation chamber. After a 10 min acclimation period, we recorded the interactions for 2 h as time-lapse movies at 1 frame  $s^{-1}$  using a digital camera (Sony DFW-X700; Sony Broadcast & Professional, Köln, Germany) in QuickTime format using SecuritySpy 2.1 (<http://www.bensoftware.com/>). We later analyzed the mating movies frame-by-frame while being blind with respect to the treatment group, to assess the number of copulations per pair. Immediately after the mating movies, we put the pairs back into well plates. On days 63 and 66, we took digital pictures of the focal GFP(+) individuals, as explained for Experiment 1, to assess body, testis, ovary, and seminal vesicle size.

From an initial 188 pairs, 32 were lost either due to incomplete development or handling errors during measurements. The final sample size was thus reduced to 156 pairs, that is on average 22.3 biological replicates per cross (range: 12–34).

We tested the effect of partner line on body, testis, ovary, and seminal vesicle size using one-way ANOVAs. Body, testis, and ovary size were log transformed and seminal vesicle size was square root transformed. Because many pairs did not copulate during the 2-h observation period, we tested whether copulation rate differed among crosses using a negative binomial regression

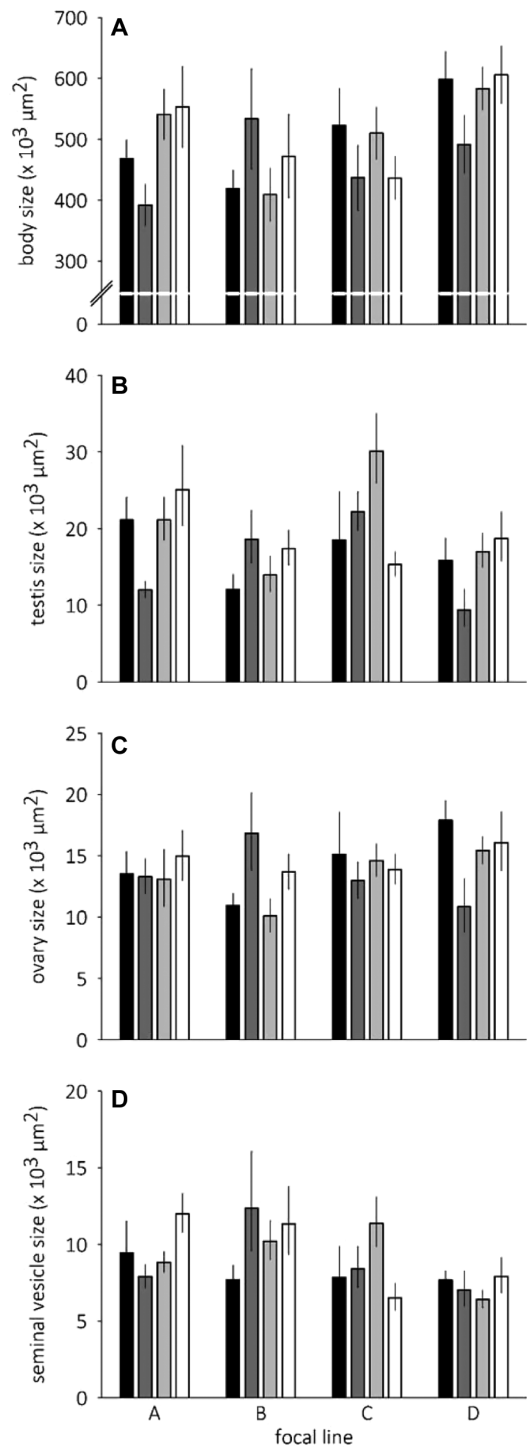
model with a maximum likelihood estimation method and a log-link function. Moreover, we investigated correlations between the four morphological traits and copulation rate using a Spearman  $r_S$  between the averaged values for each cross. Note that because the level of biological replication of the latter test only represents the seven partner lines, one should consider this test as only explorative.

### EXPERIMENT 3: REPRODUCTIVE BEHAVIOR

To test for genetic effects on copulatory and postcopulatory behaviors, we observed the interactions between paired individuals using eight outbred lines, or more specifically, eight pairs of recombinant crosses between 16 inbred lines. We first sampled F0 juvenile individuals from 16 GFP(+) inbred lines to form replicate recombinant pairs. These pairs were then regularly transferred to fresh algae, and produced offspring that were expected to be outbred and (nearly) genetically identical within each type of recombinant cross. These F1 offspring were used as experimental individuals.

On day 1, we sampled F1 offspring from each pair. Some pairs did not produce offspring, and we therefore sampled either one ( $N = 532$ ) or two ( $N = 118$ ) F1 offspring per pair, and maintained them in isolation until they reached sexual maturity. These individuals were then crossed, in a  $4 \times 4$  full factorial design, in which we a priori designated four lines as focals. Each cross consisted of two individuals that could be visually distinguished from each other during the mating trial by dyeing the focal individual 24 h before mating with a vital dye (Patent blue V, 0.25 mg/mL, Werner Schweizer AG), following the protocol described in Marie-Orleach et al. (2013).

Mating trials occurred between days 28–34, as already explained for Experiment 2. The mating trials were filmed for 2 h, and included eight replicates per mating chamber. The resulting mating movies allowed us to assess the time to first copulation, the average copulation duration in the pairs and the number of sucks performed by each individual. We restricted the observation window to the first five copulations as in Marie-Orleach et al. (2013). In addition, we tried to assess the identity of the individual that terminated each of the first five copulations in a pair. In *M. lignano*, copulating individuals form a shape that resembles two interlocking “G”s. At times, copulation termination appears to be triggered by one individual attempting to disengage itself from copula (see Fig. 5 in Schärer et al. 2004), and we could assign the identity of the individual that tried to terminate the copulation for 53% of the copulations. We then performed a principal component analysis (PCA) on the number of terminations observed by the focal and the partner individual. This approach allowed us to capture (i) the overall number of terminations observed within a pair (i.e., likely reflecting the level of conflict between mating partners over termination), and (ii) whether the terminations were



**Figure 1.** Effects of focal and partner lines on the expression of morphological traits. Graphs show (A) body size, (B) testis size, (C) ovary size, and (D) seminal vesicle size expressed by individuals of the four focal lines (line A: DV6 × LM6, line B: DV12 × LM12, line C: DV20 × LM20, and line D: DV33 × LM33) when raised with individuals of the four partner lines (DV10 in black, DV25 in dark gray, DV37 in gray and DV81 in white). Error bars indicate means  $\pm$  1SE. Values of transformed data are back-transformed to their original scale for plotting, leading to asymmetrical standard errors. See Results and Table 1 for statistics.

**Table 1.** Effects of focal line, partner line, and their interaction on the expression of morphological and behavioral reproductive traits.

	Focal line	Partner line	Interaction
<b>Exp. 1. Reproductive morphology</b>			
Body size	$F_{3,152} = 4.58, P = 0.004$	$F_{3,152} = 0.89, P = 0.447$	$F_{9,152} = 1.50, P = 0.153$
Testis size <sup>1</sup>	$F_{3,152} = 4.12, P = 0.008$	$F_{3,152} = 2.32, P = 0.078$	$F_{9,152} = 2.92, P = 0.003$
Ovary size <sup>2</sup>	$F_{3,152} = 1.01, P = 0.391$	$F_{3,152} = 0.55, P = 0.650$	$F_{9,152} = 1.53, P = 0.142$
Seminal vesicle size <sup>1</sup>	$F_{3,152} = 4.23, P = 0.007$	$F_{3,152} = 0.53, P = 0.661$	$F_{9,152} = 1.99, P = 0.044$
<b>Exp. 2. Reproductive morphology and behavior</b>			
Body size <sup>1</sup>	na	$F_{6,149} = 1.17, P = 0.325$	na
Testis size <sup>1</sup>	na	$F_{6,149} = 3.68, P = 0.002$	na
Ovary size <sup>1</sup>	na	$F_{6,149} = 0.94, P = 0.467$	na
Seminal vesicle size <sup>2</sup>	na	$F_{6,149} = 2.42, P = 0.029$	na
Copulation rate	na	Wald $\chi^2_6 = 12.29, P = 0.056$	na
<b>Exp. 3. Reproductive behavior</b>			
Time to first copulation <sup>2</sup>	$F_{3,301} = 3.53, P = 0.015$	$F_{3,301} = 7.04, P < 0.001$	$F_{9,301} = 2.67, P = 0.005$
Copulation duration <sup>1</sup>	$F_{3,301} = 4.90, P = 0.002$	$F_{3,301} = 1.49, P = 0.216$	$F_{9,301} = 0.66, P = 0.742$
Termination PC1	$F_{3,301} = 6.64, P < 0.001$	$F_{3,301} = 1.87, P = 0.134$	$F_{9,301} = 0.32, P = 0.968$
Termination PC2	$F_{3,301} = 0.85, P = 0.469$	$F_{3,301} = 0.99, P = 0.396$	$F_{9,301} = 0.64, P = 0.759$
Sucks (focal)	$\chi^2_3 = 59.46, P < 0.001$	$\chi^2_3 = 11.07, P = 0.011$	$\chi^2_9 = 5.96, P = 0.744$
Sucks (partner)	$\chi^2_3 = 7.32, P = 0.062$	$\chi^2_3 = 14.55, P = 0.002$	$\chi^2_9 = 5.91, P = 0.749$

<sup>1</sup>Log transformed.

<sup>2</sup>Square root transformed.

Results of ANOVAs are shown with associated  $F$ -tests, and GLMs with  $\chi^2$ -tests or Wald  $\chi^2$ -tests. Termination PC1 corresponds to the bias in termination between the focal and the partner, and termination PC2 corresponds to the overall number of copulation terminations that we observed in a pair (see also Fig. S2). The focal line and interaction effects are not available in Exp. 2 because we here used only a single focal line. Significant effects are indicated in bold. See methods for details.

biased toward one or the other individual in a pair, as represented by the two principal components axes (in contrast to the original two axes, see Fig. S2 for a visualization). The mating movies were analyzed blind with respect to treatments.

From an initial 384 pairs, we lost 67 replicates either because individuals did not develop properly, due to pipetting errors during pair assemblage, or because pairs copulated fewer than five times during the mating trial. The final sample size was thus reduced to 317, that is on average 19.8 pairs per cross (range: 17–23).

We analyzed time to first copulation, average copulation duration, and the two principal component axes on copulation termination using two-way ANOVAs, testing the effects of focal line, partner line, and their interaction. Time to first copulation and average copulation duration were square-root and log transformed, respectively. It is important to note that because time to first copulation, copulation duration, PC1 and PC2 are interacting phenotypes, that is traits that are compounded by both partners, the effect of the focal line on these traits essentially has the same meaning as the effect of the partner line. Therefore, any discrepancies between the focal line and partner line effects are due to random differences in the variation among the specific lines that were considered as focal lines compared to those considered as partner lines. Furthermore, we tested the effects of the focal line, the partner line, and their interaction on the number of sucks

performed by the focal and partners, separately, using generalized linear models (GLMs) with a Poisson error distribution, a log-link function and a correction for overdispersion. In addition, we investigated the correlation between the numbers of sucks performed by each partners in a pair by estimating the Spearman's rank correlation coefficient.

All statistical analyses were performed in JMP 11.0.0 (SAS Institute Inc., Cary, NC, USA).

## Results

### EXPERIMENT 1: REPRODUCTIVE MORPHOLOGY

We observed both direct and indirect genetic effects on the expression of several morphological traits. Specifically, body size differed significantly among focal lines, indicating direct genetic effects, but was not affected by the line of the partner individual or by the focal line  $\times$  partner line interaction (Fig. 1A, Table 1). Testis size differed among focal lines and, importantly, was also influenced by the line of the partner individual, as we detected a statistical trend for an effect of the partner line and a highly significant interaction effect (Fig. 1B, Table 1). Remarkably, mean testis size varied up to 2.4-fold in response to the line of the partner individual (i.e., compare the estimates of line A when paired to the “dark gray” line DV25 and to the “withe” line DV81, Fig. 1B).

**Table 2.** Interaction effect coefficients ( $\psi$ ) for morphological traits.

		Partner traits			
		Body size <sup>2</sup>	Testis size <sup>1</sup>	Ovary size <sup>1</sup>	Seminal vesicle size <sup>1</sup>
<b>Focal traits</b>	<b>Body size</b>	$\Psi = 0.153 \pm 0.127$ $P = 0.229$ interaction: $P = 0.978$	<b><math>\Psi = 0.264 \pm 0.131</math></b> <b><math>P = 0.046</math></b> interaction: $P = 0.318$	$\Psi = 0.034 \pm 0.104$ $P = 0.748$ interaction: $P = 0.104$	$\Psi = 0.001 \pm 0.100$ $P = 0.993$ interaction: $P = 0.131$
	<b>Testis size<sup>1</sup></b>	<b><math>\Psi = 0.323 \pm 0.125</math></b> <b><math>P = 0.011</math></b> interaction: $P = 0.965$	$\Psi = 0.192 \pm 0.129$ $P = 0.139$ interaction: $P = 0.571$	$\Psi = 0.030 \pm 0.102$ $P = 0.774$ interaction: $P = 0.356$	$\Psi = -0.064 \pm 0.098$ $P = 0.516$ <b>interaction: <math>P = 0.015</math></b>
	<b>Ovary size<sup>2</sup></b>	$\Psi = 0.024 \pm 0.136$ $P = 0.857$ interaction: $P = 0.696$	$\Psi = 0.054 \pm 0.140$ $P = 0.699$ interaction: $P = 0.392$	$\Psi = 0.127 \pm 0.111$ $P = 0.259$ interaction: $P = 0.274$	$\Psi = 0.055 \pm 0.107$ $P = 0.607$ interaction: $P = 0.222$
	<b>Seminal vesicle size<sup>1</sup></b>	$\Psi = 0.042 \pm 0.129$ $P = 0.743$ interaction: $P = 0.329$	$\Psi = -0.035 \pm 0.133$ $P = 0.795$ interaction: $P = 0.551$	$\Psi = 0.182 \pm 0.106$ $P = 0.0089$ <b>interaction: <math>P = 0.010</math></b>	$\Psi = -0.070 \pm 0.102$ $P = 0.494$ interaction: $P = 0.061$

<sup>1</sup>Log transformed.<sup>2</sup>Square root transformed.

$\psi$  indicates the magnitude and direction of the relationship between the traits expressed by the focal compared to the social partner. The interaction indicates whether  $\psi$  is similar across the four focal lines, and thus tests whether  $\psi$  exhibits genetic variation. All focal and partner traits were standardized (i.e., mean = 0, and SD = 1). Significant effects are indicated in bold typeface and are illustrated in Fig. 2.  $\psi$  values are estimated through a multiple linear regression for each focal trait (Moore et al. 1997). See methods for details, and Table S1 for the summary statistics.

In contrast, we observed no genetic effects on ovary size (Fig. 1C, Table 1). Finally, and similar to the result observed for testis size, seminal vesicle size showed a significant focal line effect and a significant interaction effect (Fig. 1D, Table 1).

The estimation of the interaction effect coefficient ( $\psi$ ) suggested that several focal morphological traits significantly covaried with partner morphological traits (Table 2 and Table S1). Specifically, body size and testis size expressed by focals significantly depended on the testis size and body size of their partners, respectively (Fig. 2A and B, Table 2). We found that  $\psi$  of focal testis size on partner seminal vesicle size (Fig. 2C), and focal seminal vesicle size on partner ovary size (Fig. 2D), were both significantly different across focal lines, as indicated by significant focal line  $\times$  partner trait interactions (Tables 2). These interactions suggest that  $\psi$  exhibits genetic variation. Ovary size was not influenced by morphological traits measured in the partner (i.e., all  $\psi$  were not significantly different from 0; Table 2).

## EXPERIMENT 2: REPRODUCTIVE MORPHOLOGY AND BEHAVIOR

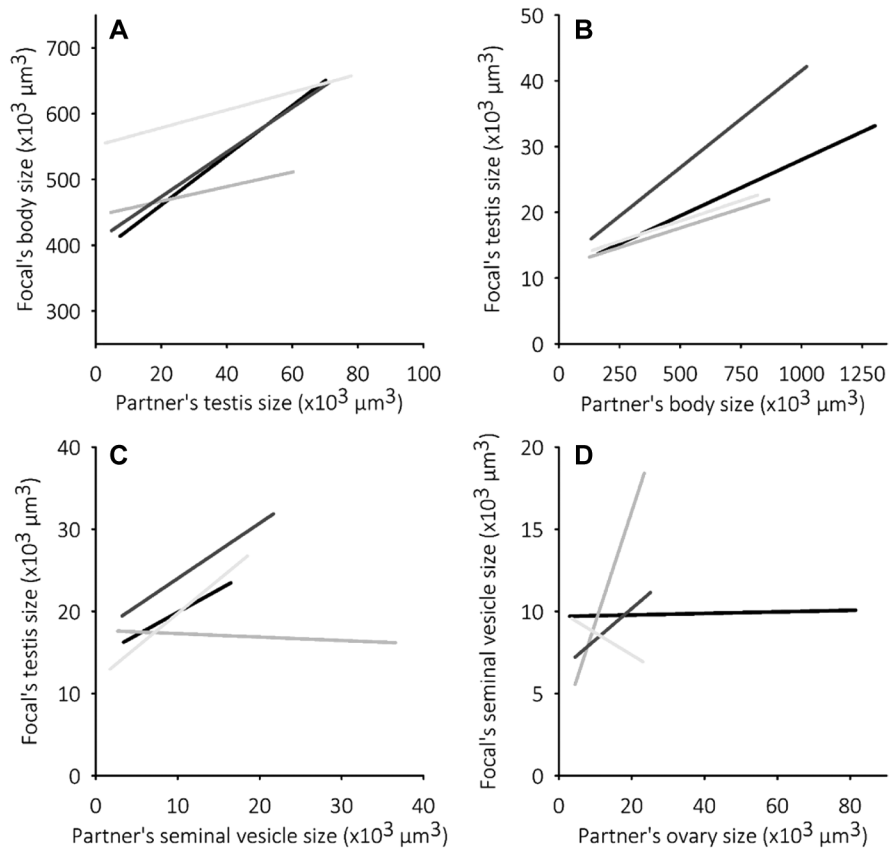
Experiment 2 largely confirmed the results found in experiment 1. Partner line strongly influenced testis and seminal vesicle size, but neither body nor ovary size (Table 1 and Fig. 3). The former varied up to 1.7-fold in response to the line of the partner individual (i.e., partner line DV29 or DV35, Fig. 3B). Copulation rate showed a trend to differ between the crosses (Table 1 and Fig. 3), and also the Spearman correlation coefficients suggested that the partner's body size ( $r_S = 0.75$ ;  $N = 7$ ;  $P = 0.052$ ) and testis size ( $r_S = 0.75$ ;  $N = 7$ ;  $P = 0.052$ ) might be positively correlated with copulation

rate, while such correlations did not seem to be present with ovary size ( $r_S = 0.464$ ;  $N = 7$ ;  $P = 0.294$ ) and seminal vesicle size ( $r_S = 0.57$ ;  $N = 7$ ;  $P = 0.180$ ). See Figure S1 for data visualization.

## EXPERIMENT 3: REPRODUCTIVE BEHAVIOR

All four behavioral traits we investigated showed significant genetic effects. Specifically, the line of both the focal and the partner individual, and their interaction, significantly affected the time to first copulation (Fig. 4A, Table 1). The size of this effect is remarkable as, for instance, individuals of focal line H started to copulate on average 1.9 times later with individuals of the "black" partner line I than with partners of the "gray" partner line K (Fig. 4A). In contrast, the average duration of the first five copulations was influenced only by the line of the focal individual (Fig. 4B, Table 1). Using a principal component analysis (PCA), we decomposed the termination behavior into PC1, explaining 71.5% of the variance and corresponding to the bias in termination between focal and partner, and PC2, explaining the remaining 28.5% of the variance and corresponding to the overall number of copulation terminations observed in a pair (see Fig. S2 for a visualization). Variation along PC1 was significantly influenced by the focal line (Fig. 4C, Table 1), but partner line had no such effects (Fig. 4D, Table 1). Together, these findings suggest that neither copulation duration nor the motivation to terminate a copulation depend on particular line combinations. The absence of a  $G \times G$  interaction in copulation termination might also explain the absence of  $G \times G$  interaction on copulation duration. Finally, the number of sucks performed by the focal individual after the first five copulations depended on the line of both the focal





**Figure 2.** Visualization of the interaction effect coefficient estimates that are significant (or have a significant interaction, see Table 2), showing linear regressions of (A) focal body size on partner testis size, (B) focal testis size on partner body size, (C) focal testis size on partner seminal vesicle size, and (D) focal seminal vesicle size on partner ovary size for each of the four focal lines: A (black), B (gray), C (dark gray), and D (light gray). See also Tables 2 and S1 for statistics.

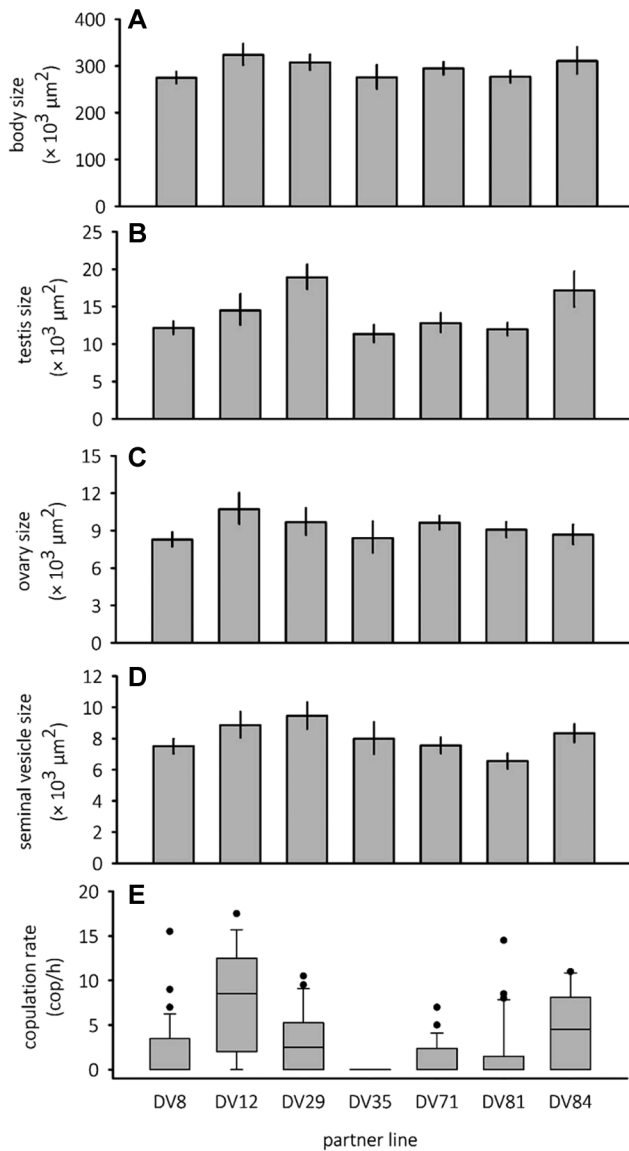
individual and its mating partner, but not on their interaction (Fig. 5A, Table 1). For instance, focal individual of line E sucked on average twice more after copulating with individuals of partner line L, compared to partner line K (Fig. 5A). This outcome was corroborated by the analysis of the number of sucks performed by the partner (Fig. 5B, Table 1), although here the effect of the focal line only showed a statistical trend.

## Discussion

Influential models of sexual conflict predict that multiple reproductive traits should show evidence of partner manipulation (Arnqvist and Rowe 2005; Moore and Pizzari 2005; González-Forero and Gavrillets 2013). By showing that several morphological and behavioral reproductive traits are influenced by the genotype of social partners in *M. lignano*, our results suggest that indirect genetic effects may play a key role in these interactions. Specifically, two independent experiments showed that testis size and seminal vesicle size are contingent on the genotype of the partners, which could be indicative of sexual conflicts over sex allocation. We further identified morphological traits expressed

in partners that may mediate such interactions, by measuring the interaction effect coefficient ( $\psi$ ), which indicated that testis size and body size of the focals depended on the body size and the testis size of the partners, respectively. Moreover, genetic variation in  $\psi$  was manifest as a  $G \times G$  interaction between partners in time to first copulation. Such an effect was not observed in copulation duration, which may be due to the absence of  $G \times G$  interactions on the motivation to terminate copulation. Finally, the occurrence of the postcopulatory suck behavior also depended on partner genotype. These empirical findings clearly demonstrate that the genotype of the partner with whom a focal has either grown up or interacted with during mating interactions, may affect the expression of multiple morphological and behavioral reproductive traits. These IGEs, observed within a generation, are expected to have several intriguing consequences for the evolutionary trajectories of these traits across generations, which we discuss in the following.

Testis size is known to be sensitive to variation in the social environment, which is argued to be an adaptive response to varying levels of sperm competition (Parker and Pizzari 2010;



**Figure 3.** Morphological traits and copulation rate of HUB1 individuals paired with partner individuals stemming from different DV lines (experiment 2). Morphological data are back-transformed to their original scale. In (A), (B), (C), and (D), error bars indicate means  $\pm$  1SE. In (E), the boxes indicate the 25<sup>th</sup>, the median, and the 75<sup>th</sup> percentile. The whiskers indicate the 10<sup>th</sup> and the 90<sup>th</sup> percentile, and the dots indicate the outliers. See Results for statistics.

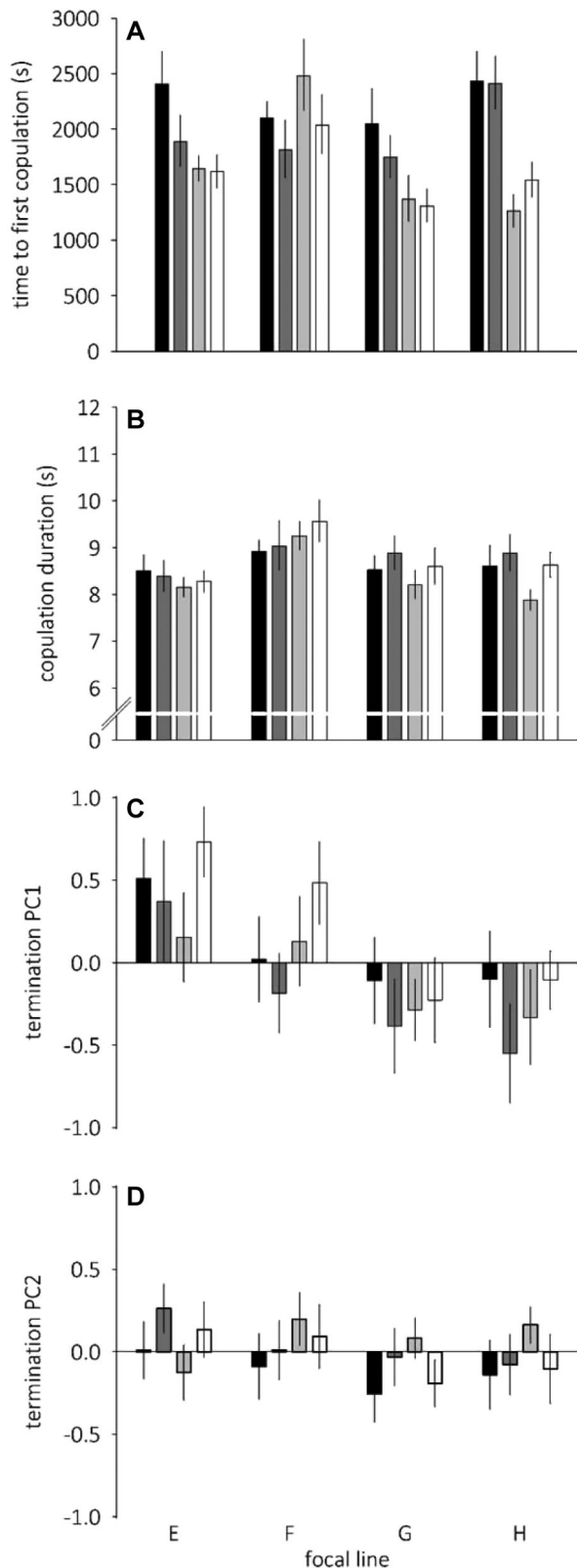
Ramm and Schärer 2014). For instance, testis size is a good predictor of the mating system in many taxa (e.g., Harcourt et al. 1981; Hosken 1997). Similarly, within species, testis size is often plastically adjusted to the social environment (e.g., Brown and Brown 2003; Tan et al. 2004; Awata et al. 2006; Firman and Simmons 2008), which has also been repeatedly shown in our model, *M. lignano* (e.g., Schärer and Ladurner 2003; Brauer et al. 2007; Janicke et al. 2013). Our findings here provide a unique illustration of how testis size is not only affected by the social

environment, but also by the genes contained in that social environment. The magnitude of this IGE is outstanding, as testis size expressed by individuals of a same focal line varied up to 2.4-fold depending on the line of the partner individual (Fig. 1B).

Variation in testis size may result from variation in the behavioral interactions experienced between mating partners. We showed that (i) some components of copulatory and postcopulatory behaviors depended on the specific combination of genotypes of the mating partners (Experiment 3, Figs. 4 and 5), and that (ii) mating rate (and thus, the likely amount of sperm expended) tended to positively correlate with testis size (Experiment 2, Fig. S1). Therefore, the amount of sperm an individual expends is expected to depend on its partner's genotype, which may feedback on sperm production rate and result in the expression of different testis sizes depending on the partner's genotype. This is consistent with the hypothesis that behavior is generally highly responsive to variation in the social environment, and may further mediate the evolution of morphological or physiological traits (Bateson 2004; Krupp et al. 2008; Bleakley et al. 2010).

An alternative and nonmutually exclusive explanation is that the partner's testis size may be manipulated by the sperm donor. In many simultaneous hermaphrodites, a sperm donor may have little interest in the male sex function of its mating partners, and selection is expected to favor the evolution of traits disrupting the partner's male sex function (Charnov 1979; Michiels 1998; Schärer 2009; Schärer et al. 2014). One possible route for such a manipulation is suggested by the fact that *M. lignano* transfers prostate gland secretions during mating (Doe 1982; Ladurner et al. 2005a; Vizoso et al. 2010), which might physiologically affect the partner's male sex function. Our finding that testis size and seminal vesicle size depended on the genotype of the partner could be indicative of genetic variation in male persistence and/or female resistance traits among the tested lines, such that sperm donors of some, but not all lines, might be able to disrupt their partner's male sex function. Fully demonstrating a sexual conflict over sex allocation in *M. lignano* would, however, require further experiments to reveal whether the observed changes in testis size incur fitness costs on the manipulated sperm recipient and fitness benefits to the manipulating sperm donor (Schärer 2014). Alternatively, the observed shifts could also represent adaptive decisions by the sperm recipient, which may not necessarily lead to fitness costs in the sperm donor.

Predicting the impact of IGEs on trait evolution may be facilitated by understanding which phenotypic traits present in the partners induce a phenotypic response in the focal individuals (Moore et al. 1997; Bleakley et al. 2010; Bailey and Hoskins 2014). The interaction effect coefficient  $\psi$  provides one way to clarify evolutionary predictions, provided that phenotypes of interest can be measured in both focal and interacting individuals (Bleakley et al.

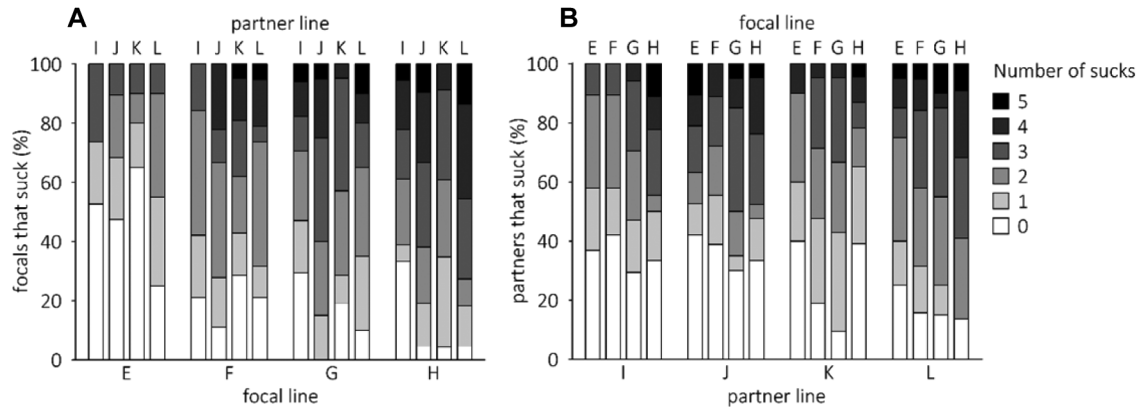


**Figure 4.** Effects of focal and partner line on copulatory behaviors. Graphs show (A) time to first copulation, (B) copulation duration, (C) termination PC1, and (D) termination PC2 measured in individuals of four focal lines (line E: LM20 × LM84; line F: LM37 × LM47; line G: LM65 × LM67; line H: LM68 × LM44) paired with

2010). However, interpreting  $\psi$  requires caution for two main reasons. First, phenotypic traits of partners might also be plastic, complicating estimation in cases where partner trait values cannot be experimentally manipulated. Second, our estimates of  $\psi$  may also capture sources of variation arising from the shared environment between mating partners, so that  $\psi$  may potentially include nongenetic components in our experimental setup. For instance, despite setting standardized conditions throughout the well plates, the positive relationship found between body size and testis size could be driven by subtle differences among the wells, leading to different conditions experienced among pairs.

Nevertheless, the significant relationships we found between the morphological traits of the focals and their partners raise several interesting questions. Particular attention has recently focused on whether individuals may in fact exhibit variation in their response to social partners (Bleakley et al. 2010; Bailey and Zuk 2012; Kazancıoğlu et al. 2012). This has stimulated interest because the amount of genetic variation in  $\psi$  indicates whether IGEs are additive, and whether  $\psi$  can itself evolve (Kazancıoğlu et al. 2012). Despite its evolutionary importance, genetic variation in  $\psi$  has, however, been investigated only in a handful of studies, all of which have detected significant variation (Kent et al. 2008; Bleakley and Brodie 2009; Chenoweth et al. 2010; Bailey and Zuk 2012). Our finding empirically demonstrates that  $\psi$  exhibits genetic variation in *M. lignano* as well. Specifically, variation in focal testis size with regards to the partner seminal vesicle size was different across focal lines. These traits capture, respectively, the sperm production and sperm usage by an individual (Schärer and Vizoso 2007). In three focal lines, focal testis size seems to vary positively with partner seminal vesicle size, which probably resulted from differences in mating rates (and thus sperm production and usage rates) among pairs. This positive relationship was, however, not observed in the fourth line used (line B in gray on Fig. 2C). Remarkably, the latter line also seemed to drive the difference observed between lines in the relationship between focal seminal vesicle size and partner ovary size, in which it showed the strongest positive relationship

individuals of four partner lines, black: line I (LM1 × LM81); dark gray: line J (LM6 × LM46); gray: line K (LM10 × LM50) and white: line L (LM12 × LM67). Termination PC1 captures the bias in copulation termination between partners: low values mean that the focal individual terminated copulations more often than the partner individual. Termination PC2 captures the overall number of copulations where an individual was identified as terminating the copulation: high values means that many copulation terminations could be observed in a pair. Error bars indicate means  $\pm$  1SE. Values of transformed data are back-transformed to their original scale, leading to asymmetric standard errors. See Results and Table 1 for statistics.



**Figure 5.** Effects of focal and partner lines on the postcopulatory suck behavior. Graphs show the percentage of (A) focal individuals and (B) partner individuals that did not suck, sucked once, twice, three, four, or five times over the first five copulations. See Results and Table 1 for statistics.

(Fig. 2D). These two significant interactions suggest that this specific line displays atypical responses to its social environment, possibly due to mating rate. However, determining which partner traits drive the observed IGEs on testis size and seminal vesicle size would require additional experiments that, for instance, use phenotypically engineered partners (Sekii et al. 2013). Together, the variation observed in  $\psi$  corroborates the  $G \times G$  interactions found on testis size and seminal vesicle size, in that they both demonstrate that the phenotypic response of an individual to its social partner exhibits genetic variation, which is a fundamental requirement for  $\psi$  to evolve (Bleakley et al. 2010; Chenoweth et al. 2010; Kazancıoğlu et al. 2012).

Copulation duration and copulation termination were affected only by the genotype of the focal individuals but not by the genotype of the mating partner or by a  $G \times G$  interaction. It is important to note, however, that for traits exclusively expressed during an interaction between mating partners (e.g., time to first copulation and copulation duration), focal genotype and partner genotype have the same biological meaning. The observed discrepancy between focal and partner genotype effects is expected to be due to sampling variation across the four lines designated as focals compared to the ones used as partners. Our results thus suggest that an individual terminates a copulation according to its own genotype and the genotype of its partner, but that the decision does not seem to depend on  $G \times G$  interactions between the mating partners. This likely explains why copulation duration is also not affected by  $G \times G$  interactions between mating partners.

Our results also indicate that lines differ in their propensity to perform the postcopulatory suck behavior, as well as in the likelihood that it is performed by their partners. The postcopulatory suck behavior has previously been hypothesized to result from a sexual conflict over the fate of the transferred ejaculate,

as this behavior is thought to remove ejaculate components out of the sperm storage organ and so to be beneficial for the sucking individuals (e.g., via ejaculate digestion, cryptic female choice, and/or limiting the risks of polyspermy) and detrimental to the sperm donor, as it removes successfully transferred ejaculate (Vizoso et al. 2010; Schärer et al. 2011; Marie-Orleach et al. 2013). Moreover, individuals tend to suck less after copulating with a virgin individual, which has previously been argued could be due to the more abundant prostate gland secretions present in virgins, whose functions might include the inhibition of the suck behavior of the sperm recipient (Marie-Orleach et al. 2013). Hence, under this hypothesis, selection would favor the coevolution of (i) persistence traits that prevent the sperm recipient from sucking, and (ii) resistance traits allowing the sperm recipient to retain control over the fate of the received ejaculate. The presence of genetic variation in the propensity to suck and to lead to sucking in the partners implies that both can respond to selection, and may coevolve under sexually antagonistic coevolution.

In conclusion, under sexual conflict, the expression of reproductive traits in one mating partner needs to depend on the genotype of the other mating partner. Our results from the free-living flatworm *M. lignano* provide evidence in support of this idea, in that we show that both morphological and behavioral reproductive traits are affected by the genotype of the mating partners. A key insight is that an individual's testis size can vary considerably depending on the genotype of its mating partners. Conflict could therefore arise over optimal allocation towards the male sex function of sperm recipients in *M. lignano*, and such an effect may be mediated by copulatory and postcopulatory interactions. Our results show that such interactions are characterised by  $G \times G$  interactions, which highlights the potential of reproductive IGEs themselves to respond to selection during periods of antagonistic coevolution.

**AUTHOR CONTRIBUTIONS**

LMO, NVB, PM, DBV and LS designed the experiments. NVB and PM performed the experiments. NVB, PM, and AS analysed the digital micrographs and mating movies. LMO and LS statistically analysed the data. LMO, NVB and LS wrote the manuscript. All authors read and approved the final version.

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**DATA ARCHIVING**

The doi for our data is 10.5061/dryad.c6d53.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Table S1.** Statistics underlying the measurement of the interaction effect coefficient,  $\psi$ .

**Figure S1.** Scatter plots of the correlations between the copulation rate and four morphological traits, including body size (A), testis size (B), ovary size (C), and seminal vesicle size (D), of HUB1 individuals paired with partner individuals stemming from seven different DV lines (experiment 2).

**Figure S2.** Bubble plot showing the number of copulations terminated by either the focal or its partner, out of the five copulations scored for each pair.