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SEX ALLOCATION ADJUSTMENT TO MATING GROUP SIZE IN A SIMULTANEOUS HERMAPHRODITE

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Sex allocation theory is considered as a touchstone of evolutionary biology, providing some of the best supported examples for Darwinian adaptation. In particular, Hamilton's local mate competition theory has been shown to generate precise predictions for extraordinary sex ratios observed in many separate-sexed organisms. In analogy to local mate competition, Charnov's mating group size model predicts how sex allocation in simultaneous hermaphrodites is affected by the mating group size (i.e., the number of mating partners plus one). Until now, studies have not directly explored the relationship between mating group size and sex allocation, which we here achieve in the simultaneously hermaphroditic flatworm *Macrostomum lignano*. Using transgenic focal worms with ubiquitous expression of green-fluorescent protein (GFP), we assessed the number of wild-type mating partners carrying GFP+ sperm from these focal worms when raised in different social group sizes. This allowed us to test directly how mating group size was related to the sex allocation of focal worms. We find that the proportion of male investment initially increases with increasing mating group size, but then saturates as predicted by theory. To our knowledge, this is the first direct test of the mating group size model in a simultaneously hermaphroditic animal.

KEY WORDS: **Local mate competition, *Macrostomum lignano*, mating group size, phenotypic plasticity, sperm competition.**

Sex allocation theory provides the theoretical framework to predict resource allocation to male and female reproduction in sexually reproducing organisms and is considered as a touchstone in evolutionary biology (Frank 2002; reviewed in Charnov 1982; Hardy 2002; West 2009). In particular, Hamilton's theory of local mate competition (LMC; Hamilton 1967) has become one of the best supported examples for Darwinian adaptation, by accurately predicting female-biased sex ratios in many separate-sexed organisms (West et al. 2000; Frank 2002).

The classic model of LMC, which is generally considered as competition between related individuals for the access to mating partners, predicts a female-biased sex ratio in spatially structured populations, where matings occur before the dispersal of females (Hamilton 1967). Specifically, there are two forces that contribute to biased sex ratios (Taylor 1981). First, the production of many sons in the same patch leads to competition among brothers for mating partners, which is not expected to be beneficial from the mother's perspective. Second, a female-biased sex ratio results in

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more mating opportunities for sons and therefore translates into a higher expected reproductive success of each produced son and an overall higher reproductive success for the mother. Only under the assumption of a large population size and random mating does LMC become negligible and only then are females expected to invest equally into sons and daughters (Hamilton 1967). The most conclusive empirical evidence for an effect of LMC on sex allocation comes from studies on parasitoid wasps, pollinating fig wasps and spider mites, which provide both qualitative and quantitative support for sex allocation theory in separate-sexed organisms (e.g., Werren 1980; Herre 1985; Macke et al. 2011; reviewed in Hardy 2002; West 2009).

The concept of LMC is also fundamental for the study of sex allocation in simultaneous hermaphrodites, that is organisms in which individuals produce male and female gametes at the same time (Charnov 1982). In contrast to separate-sexed organisms, sex allocation theory for simultaneous hermaphrodites provides the theoretical framework to predict the optimal relative investment into the male versus the female sex function within the same individual (reviewed in Schärer 2009). One central prediction of sex allocation theory for simultaneous hermaphrodites is that individuals are expected to reallocate their resources toward the female sex function if the mating group size (defined as the average number of mating partners plus one) is small (Charnov 1980, 1982). In analogy to the phenomenon of LMC in structured populations of separate-sexed organisms, a small mating group leads to competition between related sperm from a donor for the fertilization of a given set of ova (recently termed "local sperm competition"; Schärer 2009), which leads to a decelerating fitness gain for additional investment into sperm production. Therefore, simultaneous hermaphrodites are expected to have a female-biased sex allocation if the mating group size is small (Charnov 1980; Fischer 1981; Charnov 1982), as this re-allocation reduces local sperm competition and allows an overall higher reproductive success for a female-biased individual.

Charnov (1980) presented a resource allocation model, which explores explicitly the relationship between mating group size and the resulting optimal sex allocation in outcrossing simultaneous hermaphrodites (herein called the "mating group size model"). This model predicts that the proportion of reproductive resources r^* devoted to the production of sperm increases with mating group size according to the equation $r^* = (K - 1)/(2K - 1)$, where K is the number of (sperm) donors that a (sperm) recipient receives sperm from (Charnov 1980; Fischer 1981; Charnov 1982). Consequently, the resource allocation to the male sex function is predicted to increase with an increasing mating group size, reaching an asymptote at $r^* = 0.5$ as mating group size $(K + 1)$ becomes very large and more and more donors compete for a recipient's eggs.

Previous empirical work on the effect of mating group size on sex allocation in simultaneously hermaphroditic animals has mainly focused on phenotypically plastic responses in sex allocation to varying group sizes (reviewed in Schärer 2009). For instance, field studies have shown that male allocation is positively related to population density, which suggests that individuals invest more resources into the male sex function if competition for mating partners is high under natural conditions (e.g., Raimondi and Martin 1991; Hart et al. 2010). Similarly, experimental studies on a broad range of simultaneously hermaphroditic animal species provide evidence that individuals invest relatively more resources into the male sex function when kept in larger groups under laboratory conditions (e.g., Trouvé et al. 1999; Schärer and Ladurner 2003; but see Koene et al. 2006; Baeza 2007). However, in all of these studies it was unknown how density and/or social group size (i.e., the number of potential mating partners within a group) actually translated into the corresponding mating group size. In the very few cases were this relationship has been evaluated, it was shown that the mating group size can be considerably smaller than the social group size, potentially rendering social group size an unreliable estimate of mating group size (e.g., Pongratz and Michiels 2003; Janicke and Schärer 2009a). This highlights the necessity of measuring the trait that is predicted to affect the sex allocation (i.e., mating group size) rather than a proxy of it (i.e., social group size) when testing Charnov's mating group size model (see also Schärer 2009). To conclude from this, our current empirical support for the effect of mating group size on sex allocation needs to be considered as only indirect, as previous studies have not provided a direct experimental test of the relationship between mating group size and sex allocation in simultaneous hermaphrodites.

Here we report a study on the relationship between mating group size and sex allocation in the simultaneously hermaphroditic flatworm *Macrostomum lignano*. Over the last decade, *M. lignano* has emerged as a highly suitable model organism for the study of sex allocation in simultaneously hermaphroditic animals (Schärer 2009; Anthes 2010). Previous studies have showed that *M. lignano* adjusts its sex allocation in response to the social group size in a phenotypically plastic way, with individuals kept in larger groups having a more male-biased sex allocation (e.g., Schärer and Ladurner 2003; Schärer et al. 2005; Brauer et al. 2007; Janicke and Schärer 2009b). Furthermore, it has been documented that worms in larger social groups have on average more mating partners (Janicke and Schärer 2009a). In this study, we raised focal worms in a range of different social group sizes and estimated the actual mating group size and the sex allocation within the same experimental setup. Using individuals from a recently established transgenic line with ubiquitous expression of green-fluorescent protein (hereafter GFP)

as focal worms, we could estimate the resulting mating group size in a biologically meaningful way. To our knowledge, this is the first direct test of Charnov's mating group size model in a simultaneously hermaphroditic animal.

Methods

STUDY ORGANISM

The free-living flatworm *M. lignano* (Macrostomorpha, Platyhelminthes) is an obligatorily outcrossing simultaneous hermaphrodite of the intertidal meiofauna of the Northern Adriatic Sea (Schärer and Ladurner 2003; Ladurner et al. 2005). Stock cultures in the laboratory are maintained at 20 °C in glass Petri dishes filled with *f*/2 medium (Andersen et al. 2005) and fed with the algae *Nitzschia curvilineata*. The worms are transparent allowing noninvasive measurement of various morphological traits, such as testis size and ovary size (Schärer and Ladurner 2003). The transparency of the worms also enable the visualization and assessment of the number of received sperm that are stored in the female sperm-storage organ (hereafter called "antrum") in vivo (Janicke et al. 2011). The antrum usually contains ≤40 sperm and the estimates of the number of stored sperm have been shown to be highly repeatable in this species (Vizoso et al. 2010; Janicke et al. 2011). Matings are always reciprocal (Schärer et al. 2004a) suggesting that an individual receives and donates sperm while copulating. As a consequence, the number of mating partners is inherently the same for both sex functions.

CULTURE LINES

This study focuses on a phenotypically plastic adjustment of sex allocation in response to mating group size. For the experiment, we used individuals obtained from two culture lines, which are both descendants of the same inbred line. This inbred line, hereafter called DV1, was initiated by crossing two virgin worms from our genetically diverse laboratory mass cultures. In the subsequent generations, the maternal offspring of only one worm of the pair was collected and later crossed with their full- or half-siblings. In particular, only two offspring were crossed during the first 15 generations (full-sib inbreeding) and three offspring from generation 16 to 24 (full- or half-sib inbreeding). Since then, always 10 offspring (moderate level of inbreeding to maintain the lines) were used to initiate the next generations. Recently, the DV1 line was used to generate a stable germ-line transmitting transgenic line expressing enhanced GFP driven by an elongation factor 1 α promotor. This was achieved by injecting a corresponding DNA construct into a single cell stage embryo (details on the construct used, its integration, and the subsequent generation of stable homozygotes will be published elsewhere; T. Demircan et al., unpubl. ms.). The transgenic HUB1 line shows ubiquitous expression of GFP, so that this protein can also be found and visualized in the sperm cells. The transparent nature of the worms therefore allows tracking the sperm of a transgenic GFP(+) worm in a non-transgenic GFP(−) recipient *in vivo* (Fig. 1; Movie S1). In this experiment we used GFP(+) worms of the HUB1 line as focals and GFP(−) worms of the DV1 line as potential mating partners. Given that both lines originate from the same line, which was inbred for many generations, we expect GFP(+) and GFP(−) worms to be genetically almost identical, except for the fact that the GFP(+) worms carry the transgenic construct. Experiments performed in our laboratory indicate that these two lines do not differ in reproductive performance (L. Marie-Orleach et al., unpubl. ms.). Moreover, a preliminary study showed that both lines are capable of adjusting their sex allocation in a phenotypically plastic way in response to social group size, as has previously been shown for our genetically diverse mass cultures (e.g., Schärer and Ladurner 2003; Schärer et al. 2005; Janicke and Schärer 2009b).

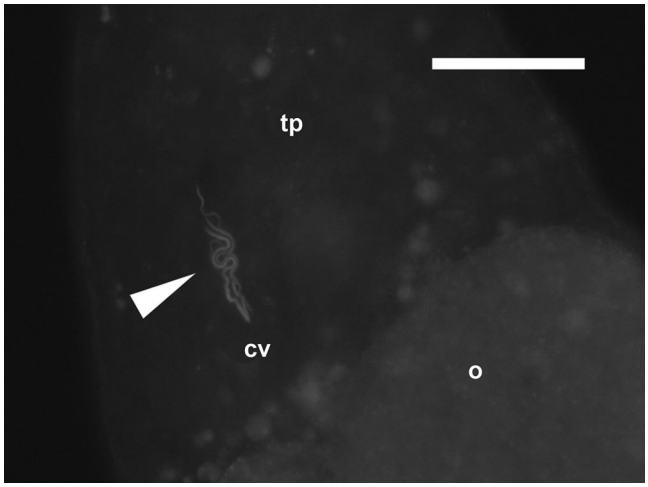

Figure 1. Micrograph of the tail region of a green-fluorescent protein (GFP)(−) worm storing four spermatozoa received from a GFP(+) worm. Image shows the GFP(+) sperm (arrowhead), the tail plate (tp), and a developed oocyte (o). Sperm are anchored with their feeler in the cellular valve (cv), which is a specialized epithelium of the sperm storage organ where the oocyte passes through before it is laid. This image is a snapshot of a monochrome movie taken under epifluorescence illumination to visualize the GFP(+) sperm (see Supporting Information). Anterior of the worm is to the bottom. Scale bar represents 50 μm.

EXPERIMENTAL DESIGN

On the first day, we collected adult worms from mass cultures of GFP(−) and GFP(+), and transferred them to glass Petri dishes filled with *f*/2 medium and a dense algae layer, to allow worms to lay eggs. In detail, we distributed 250 adult GFP(+) worms equally among two Petri dishes and 1800 adult GFP(−) worms equally among 20 Petri dishes. On day 4, all adult worms were

removed from the Petri dishes so that all eggs were laid within 72 h, which guaranteed that all offspring produced were of similar age and stemmed from parents held in very similar backgrounds. On day 15, we pooled all offspring produced by GFP(+) and GFP(−) worms respectively, and distributed them randomly among the treatment groups. Specifically, we transferred one GFP(+) focal worm to wells of 24-well tissue well-plates (TPP AG, Switzerland) and added to each focal a specific number of GFP(−) worms so that the final social group size was 2, 3, 4, 5, 8, 12, or 16 worms (e.g., groups of eight individuals consisted of one GFP(+) worm and seven GFP(−) worms). All wells contained 1.5 mL of *f*/2 medium and a standard amount of an algae solution that guaranteed ad libitum food conditions (i.e., a dense layer of algae on the bottom of the wells). We arranged the treatments on the well-plates in a way that balanced any potential position effects. Initially, we replicated all social group sizes 20 times so that the experiment comprised overall 140 GFP(+) worms and 860 GFP(−) worms. On days 22, 35, 47, and 55, all worms were transferred to fresh wells (i.e., 1.5 mL *f*/2 medium and a dense algae layer) to guarantee continued ad libitum food conditions and to reduce possible interactions of adult worms with their offspring.

ESTIMATION OF SEX ALLOCATION AND MATING GROUP SIZE

From day 62 to day 69, we took morphological measurements of the GFP(+) focals and assessed the presence of GFP(+) sperm in the antrum of each of the GFP(−) worms within each social group (Fig. 1). At the same time, we also checked whether all the worms within a social group were mature, as inferred from a full development of the gonads and the male copulatory organ. To avoid time effects, we balanced the treatment groups sampled among days. Specifically, we first isolated all individuals of a given social group in wells of 60-well microtest plates (Greiner Bio-One, Germany) filled with 10 μL of *f*/2 medium. We did this to prevent gradual changes in the composition in a social group as such changes could potentially affect the sperm representation of the focal worms. Next, we identified the GFP(+) focal of each social group using a MZ12.5 stereo-microscope equipped with a epifluorescence light source (Leica Microsystems, Germany) and then took pictures for morphometry following a standard protocol with a compound microscope (Schärer and Ladurner 2003). In brief, focals were anesthetized in a 5:3 mixture of 7.14% $MgCl_2$ and *f*/2 medium for 10 min. Thereafter, we squeezed focals dorsoventrally to a fixed thickness of 35 μm between a microscope slide and a cover slip of a hemocytometer, and took digital micrographs of the entire body, the testes, and the ovaries with a Leica DM 2500 microscope (Leica Microsystems) and a digital video camera (DFK 41AF02, The Imaging Source Europe GmbH, Germany; 40× magnification for body size and 400× magnification for testis size and ovary size). For image acquisition, we used BTV Pro 6.0b1 (http://www.bensoftware.com/) and we analyzed micrographs using ImageJ 1.42k (http://rsb.info.nih.gov/ij/). All these morphological measurements have been shown to have a high repeatability (Schärer and Ladurner 2003).

We further assessed the presence of stored GFP(+) sperm in each of the GFP(−) worms based on movies of the antrum, which were recorded as described previously (Janicke et al. 2011). Briefly, we compressed anesthetized worms between a 24 mm × 50 mm and a 21 mm × 26 mm cover slip using small plasticine feet as spacers. Afterward, we mounted this cover slip chamber on a microscope slide, so that the observer could easily flip the worm from the dorsal to the ventral view, allowing accurate assessment of the presence of sperm stored in the antrum (see Janicke et al. 2011). We recorded movies of each antrum by focusing slowly through the entire organ at a 630× magnification under epifluorescence illumination to visualize the GFP(+) sperm transferred by the GFP(+) focal (Movie S1). For this we used a Leica DM 2500 microscope (Leica Microsystems) equipped with an epifluorescence light source and connected with a highly sensitive digital video camera, a Leica DFC 360 FX (Leica Microsystems). Movies were recorded using the screen-capture software CamStudio version 2.0 (http://camstudio.org) and analyzed using KMPlayer version 3.0 (http://kmplayer.com/forums).

Based on these movies we assessed the presence of stored GFP(+) sperm in the antrum of GFP(−) worms. Mating group size was assessed as the number of GFP(−) individuals in the social group that had at least one GFP(+) sperm in storage plus one so that the mating group includes the number of mates of a given focal individual and the focal individual itself (cf. Charnov 1982).

We need to clarify here that our estimate of mating group size does not necessarily reflect the actual number of mating partners that a focal individual has had over a certain time span, because it relies exclusively on the current presence of successfully stored sperm in its partners. Processes associated with the removal of transferred sperm (e.g., sperm displacement, passive sperm loss, and/or cryptic-female choice) or the usage of sperm for fertilizing the eggs will lead to an underestimation of the number of mating partners (see also Janicke and Schärer 2009a), so that the total number of mating partners of the focal worms over the period of the experiment was presumably higher than our results suggest. However, the crucial trait predicted to affect the sex allocation in simultaneous hermaphrodites is not the total number of mates, but the average number of mating partners that are in competition for a given set of ova (Charnov 1982; Schärer 2009; cf. Parker 1998). Our measurement of the number of mating partners, which is based on the presence of stored sperm, corresponds to the number of mating partners in Charnov's mating group size model (termed

"*K*" in the original equation; see Introduction) and is therefore an appropriate estimate of the mating group size in the context of sex allocation theory.

STATISTICAL ANALYSES

From the intended total sample size of 140 replicates we lost 56 replicates, mainly because some worms did not develop properly or grew slowly. Given that the worms used for this experiment originated from an inbred line this is not surprising and matches with our experience with this and other inbred lines we are maintaining. Specifically, we lost 40 replicates due to incomplete development of either the focal or one or more of its partners (e.g., lack of the testes, ovaries, and/or male copulatory organ), 11 replicates due to pipetting errors during transfers, and five replicates due to handling errors during morphological measurements. Consequently, our final sample size was reduced to 84 replicates (pairs: $N = 16$, triplets: $N = 13$, quartets: $N = 14$, quintets: $N = 15$, octets: $N = 10$, groups of 12 worms: $N = 10$, groups of 16 worms: $N = 6$; incomplete development of individual worms is of course more likely to affect the larger social groups).

In this study, we used the proportion of testis size to overall gonad size (i.e., testis size/[testis size + ovary size]) as an estimate of sex allocation (cf. Vizoso and Schärer 2007; Janicke and Schärer 2009b). We note that this estimate represents a relative measure of the sex allocation, which allows comparing the resource allocation toward the male and female sex function between individuals, with higher values indicating a more male-biased sex allocation. However, it does not provide an absolute measure of sex allocation, because it is exclusively based on measures of the size of male and female gonadal tissues, which, although both involved in gamete production do not necessarily equal in terms of energetic demands per unit size. Furthermore, additional traits that may also impose costs to male and female reproduction (e.g., copulatory organs, seminal fluids, yolk, egg-shell glands, sex-specific behaviors) are not considered here (cf. Schärer and Pen 2013). Consequently, our estimate of sex allocation relies on the assumption that testis and ovary size are good proxies for the reproductive investment into the male and female sex function, respectively (reviewed in Schärer 2009). For *M. lignano* this assumption has been verified directly for testis size (e.g., Schärer et al. 2004b; Schärer and Vizoso 2007), whereas evidence that ovary size reflects the resource allocation into the female sex function is less direct (e.g., Schärer et al. 2005).

The statistical test of Charnov's mating group size model was done in the following two steps. First, we tested the effect of our experimental manipulation of the social group size on sex allocation and on mating group size of the GFP(+) focals. Second, we explored the relationship between our estimates of mating group size and sex allocation among social group sizes to test Charnov's mating group size model.

Effect of social group size on sex allocation and mating group size

First, we tested whether the social group size affected the body size of focal worms using a Kruskal–Wallis rank sum test. This was done to infer whether the overall resource budgets differed between the social groups. In this study, we were primarily interested in effects on sex allocation, but for a more complete data representation we also tested whether social group size affected testis size and ovary size independently (as suggested by Schärer 2009) using Kruskal–Wallis rank sum tests. Finally, we tested whether social group size had an effect on the sex allocation and on the mating group size using Kruskal–Wallis rank sum tests. Post hoc tests were conducted using Wilcoxon rank sum tests with Benjamini–Hochberg adjustment of P values to correct for false discovery rates (Benjamini and Hochberg 1995). We used nonparametric tests to account for unequal variances across treatment groups and/or deviations from normality.

Test of Charnov's mating group size model

We fitted linear and quadratic regressions to explore how sex allocation relates to the mating group size. Quadratic regressions were applied to account for the fact that Charnov's mating group size model predicts that the relationship between sex allocation and mating group size is nonlinear. We ran log-likelihood ratio tests and obtained the Akaike information criterion (AIC) to evaluate whether the nonlinear model provides a better fit than the linear model. First, we fitted a linear and a quadratic regression on the arithmetic means of sex allocation and mating group size computed separately for each social group size. This was done to relate the experimentally induced variation in mating group size to the experimentally induced variation in sex allocation. We weighted these mean values in both models according to the number of replicates obtained for each social group size, to account for differences in the accuracy of our estimates. Second, we similarly fitted and compared linear and quadratic regressions on the individual data to provide a largely descriptive test of how individual variation in mating group size translates into sex allocation (i.e., only part of this variation is due to our experimental manipulation).

All statistical analyses were carried out in R version 2.15.2 (R Development Core Team 2012). Values are given as mean ± 1 SE.

Results

Social group size had an effect on the body size of focal worms (Kruskal–Wallis rank sum test: $\chi^2 = 33.38$, degrees of freedom [df] = 6, $P < 0.001$). Focal worms kept in larger social groups grew bigger (Fig. 2A) suggesting that individuals kept in larger social groups had an overall higher resource budget compared to

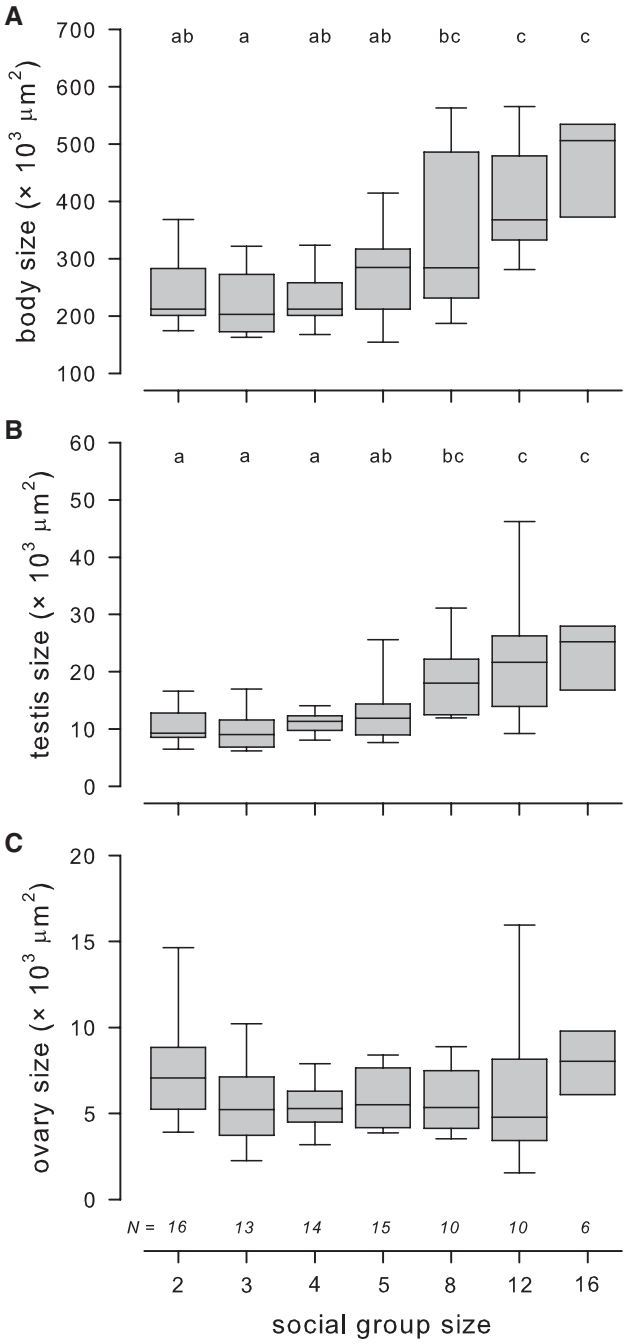

Figure 2. Effects of social group size on (A) body size, (B) testis size, and (C) ovary size. Different letters indicate significantly different treatment groups inferred from Wilcoxon rank sum post hoc tests (corrected for multiple testing, see main text). Boxplots show the 25th percentile, the median, and the 75th percentile and whiskers denote the 5th and the 95th percentiles.

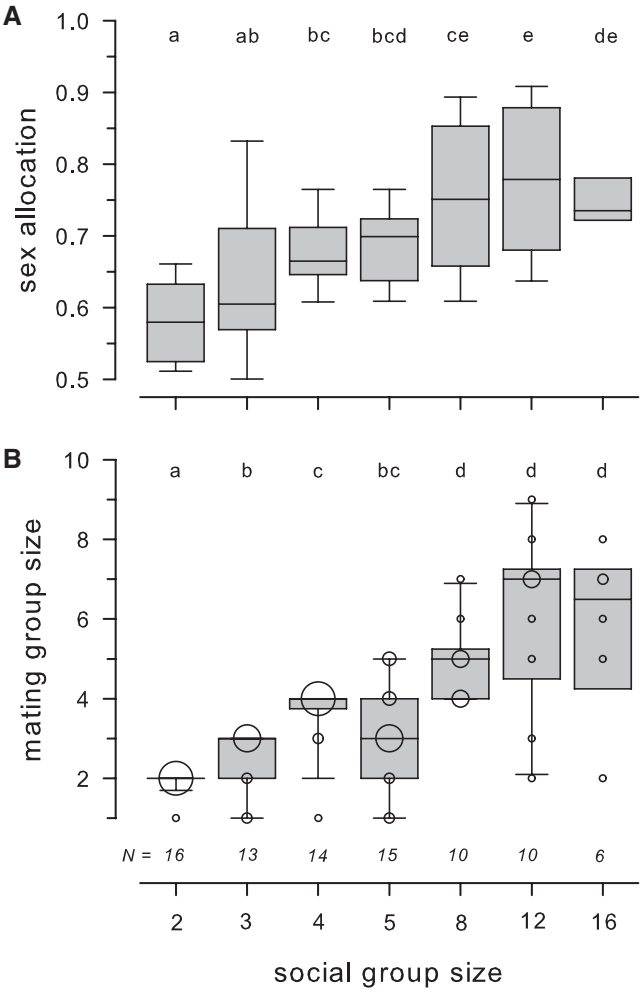

Figure 3. Effects of social group size on (A) sex allocation (i.e., testis size divided by overall gonad size) and (B) mating group size (i.e., the number of partners carrying green-fluorescent protein [GFP](+) sperm plus one). Different letters indicate significantly different groups inferred from Wilcoxon rank sum post hoc tests (corrected for multiple testing, see main text). Boxplots show the 25th percentile, the median, and the 75th percentile and whiskers denote the 5th and the 95th percentiles. Open circles in (B) are individual data points and circle size reflects the number of cases for which we observed a given mating group size.

individuals kept in smaller social groups. Individuals of different social groups varied significantly in testis size (Kruskal–Wallis rank sum test: $\chi^2 = 36.96$, df $= 6$, $P < 0.001$; Fig. 2B) but not in ovary size (Kruskal–Wallis rank sum test: $\chi^2 = 10.68$, df $= 6$, $P = 0.099$; Fig. 2C). As a consequence, social group size had a strong effect on the sex allocation of focal worms (Kruskal–Wallis rank sum test: $\chi^2 = 38.36$, df $= 6$, $P < 0.001$). Specifically, individuals raised in larger groups had a more male-biased sex allocation compared to individuals in smaller groups (Fig. 3A).

Our manipulation of the social group size also induced considerable variation in the mating group size (Kruskal–Wallis rank sum test: $\chi^2 = 48.88$, df $= 6$, $P < 0.001$), in that focal worms in larger groups managed to store sperm in more partners than individuals in smaller groups (Fig. 3B). Interestingly, the number of mating partners leveled off with increasing social

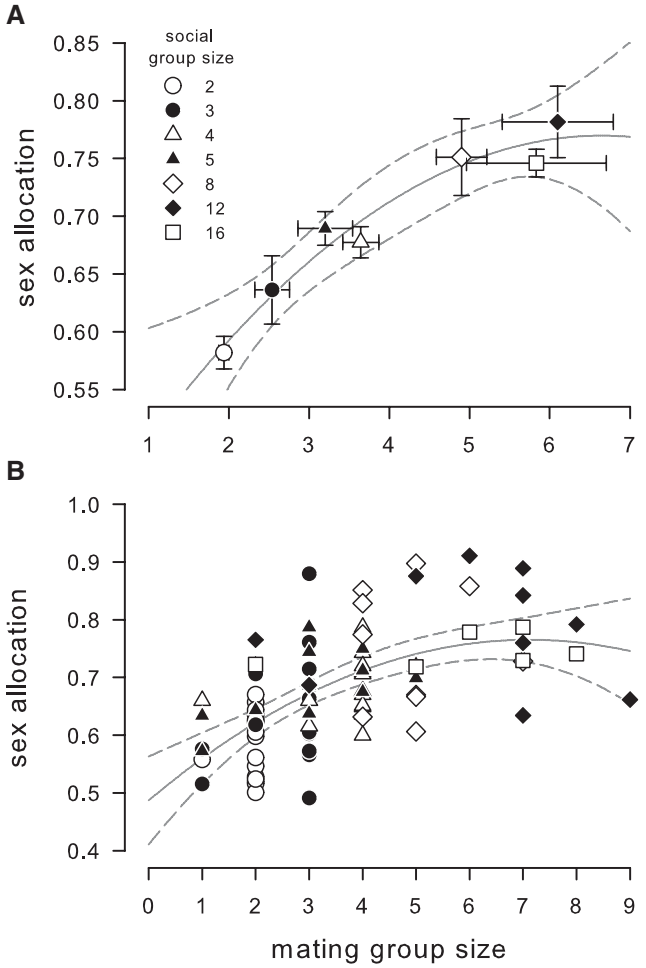

Figure 4. Relationship between sex allocation and mating group size shown for (A) group means ± 1 SE of sex allocation and mating group size obtained from each social group size and (B) individual data points. Symbols indicate the corresponding social group size (see legend). Solid line shows the fit of a quadratic regression (model fit on weighted means: $y = 0.70 + 0.17x - 0.04x^2$; model fit on individual data points: $y = 0.68 + 0.51x - 0.21x^2$), dashed lines indicate the 95% confidence intervals. Note that statistical tests on the group means were done on weighted means and that the SEs are only shown as a visual aid (see Methods section).

group size (i.e., individuals in social groups of 8, 12, and 16 did not differ in mating group size; Fig. 3B).

Mean estimates of sex allocation were clearly positively related to mean estimates of mating group size obtained from each social group size (linear regression: $R^2 = 0.92$, $F_{1,5} = 56.25$, $P < 0.001$; quadratic regression: $R^2 = 0.96$, $F_{2,4} = 51.05$, $P = 0.001$) with the quadratic regression providing a significantly better fit than the linear regression (likelihood ratio test: $\chi^2 = 5.41$, df $= 1$, $P = 0.020$; $AIC_{\text{linear regression}} = -29.53$; $AIC_{\text{quadratic regression}} = -32.94$). Sex allocation increased with increasing mating group size in a saturating manner (Fig. 4A). Very similar patterns were found in the descriptive analysis, in which we tested how individual variation in mating group size translated into sex allocation using individual data points (linear regression: $R^2 = 0.31$, $F_{1,82} = 37.49$, $P < 0.001$; quadratic regression: $R^2 = 0.37$, $F_{2,81} = 23.41$, $P < 0.001$; likelihood ratio test: $\chi^2 = 6.70$, df $= 1$, $P = 0.010$; $AIC_{\text{linear regression}} = -176.91$; $AIC_{\text{quadratic regression}} = -181.61$; Fig. 4B).

Discussion

This study provides the first direct test of Charnov's mating group size model for a simultaneously hermaphroditic animal. First, we show that experimental manipulation of the social group size induces variation in both sex allocation and mating group size, which confirms earlier results obtained in separate studies. Second, we demonstrate, to our knowledge for the first time, that sex allocation and mating group size are positively related in a saturating manner, as predicted by sex allocation theory. In the following we discuss these two major outcomes in more detail.

EFFECTS OF SOCIAL GROUP SIZE ON SEX ALLOCATION AND MATING GROUP SIZE

Our results confirm earlier findings on the effect of social group size on sex allocation in *M. lignano*, which have also shown that worms in larger groups have a more male-biased sex allocation (e.g., Schärer and Ladurner 2003; Schärer et al. 2005; Brauer et al. 2007; Janicke and Schärer 2009b). In contrast to these previous studies, in which the social group size ranged only from 2 to 10 individuals, we here also tested social groups of 12 and 16 individuals, with the intention to explore whether the sex allocation adjustment continues or whether it reaches an asymptote. Interestingly, we found that the sex allocation of individuals kept in groups of 12 and 16 individuals did not differ from that of individuals kept in octets. This suggests that the previous studies had probably already covered the maximum variation in sex allocation that can be observed in *M. lignano* as the result of a phenotypically plastic response to differences in social group size, at least under laboratory conditions. Future studies should clearly try to assess the mating group size in the field to get an idea about the natural variation in mating group size and how it translates to estimates obtained under laboratory conditions.

We also found a strong effect of social group size on the body size of the worms, which has been previously found in some, but not all studies on plasticity of sex allocation in *M. lignano* (e.g., Schärer and Janicke 2009; but see Janicke and Schärer 2009b). In theory, this finding could have complicated our conclusions about the effect of social group size on sex allocation, because body size itself has been argued to affect the sex allocation in simultaneous hermaphrodites (reviewed in Schärer 2009). In accordance with that prediction, there is evidence for such a size-dependent sex

allocation in *M. lignano*, with smaller individuals having a more male-biased sex allocation when kept in the same group size (Vizoso and Schärer 2007). However, in our study, individuals in larger groups grew bigger and had a more male-biased sex allocation, which is exactly the opposite of what is predicted by theory on size-dependent sex allocation. Therefore, we believe that size-dependent sex allocation is unlikely to explain the observed effect of social group size on the resource allocation into the male and the female sex function. Nevertheless, given the observed positive effect of social group size on body size and the presence of size-dependent sex allocation in *M. lignano*, it remains possible that we might have underestimated the variation in sex allocation in this study.

We need to clarify here that our measure of sex allocation does not represent an absolute but only a relative estimate of the resource allocation devoted to the male versus the female sex function. This is because sex allocation was measured in terms of the size of the gonadal tissue rather than in terms of the energy invested into both sex functions. As a consequence, estimates greater than 0.5 (cf. Fig. 3A) are not necessarily indicative of a male-biased sex allocation (see also Methods section). Instead, our estimate of sex allocation only provides a relative measure, which still allows us to compare changes in resource allocation toward the male and the female sex function between individuals (reviewed in Schärer 2009).

Social group size was also found to affect our estimate of mating group size. As expected, focal worms that were kept in larger social groups managed to store sperm in more mating partners. Interestingly, we found no difference in the number of mating partners between social groups of 8, 12, and 16 individuals suggesting that there is an upper threshold in the number of individuals that can be successfully inseminated by a focal worm. The average numbers of mating partners found in this study correspond largely to the results of an earlier study in which sperm-labeled focal worms were kept in social groups of 2, 3, 4, 8, and 16 individuals (using an older sperm-labeling technique; Janicke and Schärer 2009a). This is somewhat surprising, because in the earlier study focal worms were allowed to mate within their social group for only 24 h and not for several weeks as in this study. We suspect that the reason why the much longer group exposure of focal worms did not lead to a higher number of successfully inseminated partners compared to the previous study, is a high turnover rate of the sperm stored in the female sperm storage organ. Especially sperm displacement and/or passive sperm loss during egg laying are likely to reduce the time that received sperm remains stored in the female sperm storage organ in *M. lignano*. Recent studies on *M. lignano* indicate that there is second male sperm precedence caused by sperm displacement (P. Sandner et al., unpubl. ms.; L. Marie-Orleach et al., unpubl. ms.). Furthermore, given that fertilized eggs have to pass through the antrum (i.e., the sperm storage organ), before being laid (Vizoso et al. 2010), it is likely that some of the stored sperm are passively lost during egg laying. In addition, active sperm removal by the recipient (e.g., cryptic-female choice) might be an additional mechanism, which limits the time that sperm remains stored (for possible mechanisms, see Vizoso et al. 2010). Finally, the usage of sperm to fertilize the eggs will also deplete the number of sperm that is stored in the sperm storage organ, which eventually also constrains the time that the sperm of a given donor remains stored in the recipient. Here it is worthwhile to note that sperm depletion due to passive sperm loss and/or sperm usage for fertilization might be particularly important in *M. lignano* as worms usually store relatively few sperm in their sperm storage organ (e.g., on average 29 sperm; Janicke et al. 2011).

Our finding that the mating group size does not exceed a certain threshold in *M. lignano* may have important implications for the evolutionary stability of simultaneous hermaphroditism. Sex allocation theory suggests that simultaneous hermaphroditism is an evolutionary stable strategy if the mating group size remains relatively small (Charnov 1982). This is because small mating group sizes and the associated high potential for local sperm competition lead to a saturating fitness curve for the male sex function, and such a saturating fitness gain curve in one sex function is a prerequisite for simultaneous hermaphroditism to be resistant against the invasion of pure males and females (reviewed in Charnov 1982; Schärer 2009). Our results suggest that the maximum average mating group size in *M. lignano* is approximately six, a range where the theoretically predicted sex allocation is 0.44 and thus well below 0.5. Further work is clearly needed to identify the mechanisms, which are causing the observed upper threshold of mating group size in *M. lignano*.

TEST OF CHARNOV'S MATING GROUP SIZE MODEL

The major novel insight of this study is the documentation of a positive and nonlinear relationship between mating group size and sex allocation, as predicted by the mating group size model (Charnov 1980, 1982). Together with empirical studies on separate-sexed organisms (e.g., Werren 1980, 1983; Herre 1985; reviewed in West 2009), our work suggests that Hamilton's LMC theory, which has later been extended to simultaneous hermaphrodites (Charnov 1980; Fischer 1981), provides valid predictions that are universal for animals of various gender expressions.

Previous empirical tests of the Charnov's mating group size model for simultaneous hermaphrodites have used social group size or density as proxies for mating group size and therefore provided only indirect support for the theory (e.g., Raimondi and Martin 1991; Schärer and Ladurner 2003; Tan et al. 2004; Janicke and Schärer 2009b; for an experimental evolution study on plants, see Dorken and Pannell 2009; reviewed in Schärer

2009). Although social group size is presumably often positively related to mating group size, data of this and a previous study (Janicke and Schärer 2009a) suggest that this relationship can be non-linear, so that social group size becomes an inaccurate estimate of mating group size. Therefore, we argue that measuring mating group size is a crucial prerequisite to provide a more direct experimental test of the mating group size model.

PERSPECTIVES

Our study is the first to directly quantify the relationship between mating group size and sex allocation in a simultaneously hermaphroditic animal. However, we have to clarify that our experimental design might still not provide the ultimate test of Charnov's mating group size model for at least two reasons.

First, the mating group sizes model makes a number of assumptions that might not accurately match the biology of our model organism. Specifically, one important assumption of Charnov's mating group size model is that the proportion of eggs that are fertilized by a donor depends only upon the number of sperm donated by that donor in relation to the number of sperm donated by other individuals (i.e., the model assumes a fair-raffle sperm competition; Charnov 1980, 1982). However, in many organisms this assumption probably does not apply, due to both random and nonrandom processes, which have been argued to bias the fraction of sperm stored from particular donors, so that also the mating group size can become an imprecise estimate for the intensity of local sperm competition (Charnov 1996; Greeff et al. 2001; Schärer and Pen 2013). Indeed, for many simultaneously hermaphroditic animals, including *M. lignano*, there is evidence for biased sperm precedence (e.g., Angeloni et al. 2003; Pongratz and Michiels 2003; Garefalaki et al. 2010; P. Sandner et al., unpubl. ms.), which ultimately leads to a skewed representation of a donor's sperm in the partners sperm storage organ. Therefore, mating group size, as generally considered and as measured here, might still underestimate the intensity of local sperm competition (and thus overestimate the effective mating group size) in our and other model organisms. Future studies on the link between local sperm competition and sex allocation should attempt to explicitly quantify the skewed representation of sperm stored by different donors in a recipient and test how such skews can affect the sex allocation in simultaneous hermaphrodites (Greeff et al. 2001; Schärer and Pen 2013).

Second, our data do not provide any information about causality as this would have required to manipulate the number of successful mating partners experimentally, which will be very difficult if not impossible to achieve in our and other model systems. As a consequence we cannot exclude alternative hypotheses that are also predicting a positive effect of group size on the sex allocation. For instance, an increased male allocation in larger groups might have been an adaptation to an increased mating rate rather than more sperm competition, as suggested by the "male mating rate hypothesis" (reviewed in Vahed and Parker 2012).

Having these two caveats in mind, we suggest that further work should focus on (1) quantifying skews in sperm transfer success and (2) on using alternative approaches to quantify the relationship between local sperm competition and sex allocation. In particular, one very promising direction would be to test sex allocation theory at a microevolutionary level. To our knowledge, there is only one experimental evolution study on separate-sexed spider mites, which demonstrates strikingly how sex ratios evolve in response to LMC (Macke et al. 2011). For simultaneously hermaphroditic animals we still lack an analogous experimental proof for the evolution of sex allocation in response to local sperm competition (but see Dorken and Pannell 2009 for plants). In addition to approaches using comparisons across species (e.g., Petersen 1991), such experimental evolution studies are clearly needed to complement the currently available empirical support for sex allocation theory in simultaneous hermaphrodites.

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Supporting Information

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

Movie S1. Movie showing the tail region of an individual that stores four sperm received from a GFP(+) worm in its sperm storage organ.