Determinants of mating and sperm-transfer success in a simultaneous hermaphrodite

T. JANICKE & L. SCHÄRER

Zoological Institute, University of Basel, Basel, Switzerland

Abstract

The number of mating partners an individual has within a population is a crucial parameter in sex allocation theory for simultaneous hermaphrodites because it is predicted to be one of the main parameters influencing sex allocation. However, little is known about the factors that determine the number of mates in simultaneous hermaphrodites. Furthermore, in order to understand the benefits obtained by resource allocation into the male function it is important to identify the factors that predict sperm-transfer success, i.e. the number of sperm a donor manages to store in a mate. In this study we experimentally tested how social group size (i.e. the number of all potential mates within a population) and density affect the number of mates and sperm-transfer success in the outcrossing hermaphroditic flatworm *Macrostomum lignano*. In addition, we assessed whether these parameters covary with morphological traits, such as body size, testis size and genital morphology. For this we used a method, which allows tracking sperm of a labelled donor in an unlabelled mate. We found considerable variation in the number of mates and sperm-transfer success between individuals. The number of mates increased with social group size, and was higher in worms with larger testes, but there was no effect of density. Similarly, sperm-transfer success was affected by social group size and testis size, but in addition this parameter was influenced by genital morphology. Our study demonstrates for the first time that the social context and the morphology of sperm donors are important predictors of the number of mates and sperm-transfer success in a simultaneous hermaphrodite. Based on these findings, we hypothesize that sex allocation influences the mating behaviour and outcome of sperm competition.

Introduction

Our understanding of how sexually reproducing organisms allocate their resources to male vs. female offspring has increased remarkably over the last decades making sex allocation theory one of the best examples for Darwinian adaptation in evolutionary biology (Klinkhamer *et al.*, 1997; Frank, 2002). However, there are still fundamental assumptions and important predictions of that theory that remain to be tested (Reece *et al.*, 2008). Regarding the reproductive strategy an organism should possess, sex allocation theory predicts that hermaphro-

Correspondence: Tim Janicke, Zoological Institute, University of Basel, Vesalgasse 1, CH-4051 Basel, Switzerland.
Tel.: +41 61 267 03 75; fax: +41 61 267 03 62; e-mail: tim.janicke@unibas.ch

ditism is favoured if the reproductive success of at least one sex function shows a law of diminishing returns, favouring the reallocation of resources from that function to the other (Charnov, 1982). This implies that the fitness increase in one sex function must be greater for the first resources allocated into it than for any further resources, which is depicted by a saturating fitness gain curve. The classical resource allocation model for simultaneous hermaphrodites by Charnov (1979) assumes that the female gain curve is linear and that the male gain curve saturates. The rationale for this is Bateman's principle, which states that the female's reproductive success is primarily limited by the energy available for producing gametes, whereas the reproductive output of males is primarily governed by the number of mates (Bateman, 1948). Therefore, the female fitness is supposed to be

proportional to the resource input into eggs and the male gain curve is expected to saturate because of limitations in male mating opportunities (Charnov, 1982). As a consequence of a lowered average number of mating partners a form of local mate competition among sperm from the same donor might arise, which decelerates the fitness gain of any additional resource devoted to the male function (Hamilton, 1967; Charnov, 1979; Fischer, 1981). As the saturation of the male gain curve occurs more rapidly when the number of mating partners is low, it follows that hermaphroditism is a stable reproductive strategy only if the average number of mates is relatively small (Charnov, 1982).

In simultaneous hermaphrodites the number of mates is usually expressed in terms of mating group size, which is defined as the number of mating partners an individual has within a reproductive period plus one (Charnov, 1982). As this term is specific for hermaphrodites, we will use 'the number of mates' instead of 'mating group size' throughout this study. However, we would like to stress that the only difference between 'mating group size' and 'the number of mates' is the fact that the former term includes the focal individual whose mating partners were counted whereas the latter does not.

Despite the central role of the number of mates in sex allocation theory, only very little is known about the absolute number of mating partners simultaneous hermaphrodites can acquire and on how this number varies between different individuals. Moreover, our knowledge about the factors that are responsible for the variation in reproductive success via each sex function is very scarce for hermaphrodites. However, this information is certainly needed to understand variation in sex allocation in simultaneous hermaphrodites, i.e. in the allocation of reproductive resources to the male vs. the female function. As outlined above, the fitness of the male function might primarily be governed by the number of mating partners (Bateman, 1948). Yet, in species where individuals are promiscuous, sperm from different individuals compete for fertilization and thus the number of offspring sired by the male function will also depend on the outcome of sperm competition (Parker, 1970). Moreover, fertilization success is not only a function of male–male competition. In many species, females are able to manipulate paternity at the pre- and/or post-copulatory level (Eberhard, 1996). There is increasing evidence that females choose among sperm from different males post-copulatorily and that this so-called cryptic female choice (Thornhill, 1983) may also affect paternity significantly (e.g. Pizzari & Birkhead, 2000; Bussière et al., 2006). Therefore, a complete understanding of the pay-offs obtained by resource allocation into the male function requires not only insights into the factors that predict the number of mating partners but also the traits that influence the number of sperm an individual is able to successfully get into storage in the partners' sperm storage organ.

Here we demonstrate for a hermaphroditic flatworm how social group size (i.e. the number of all potential mates within a population) translates into the number of mates and we identify a number of key factors that determine the number of mates and sperm-transfer success – an issue that has never been addressed explicitly in simultaneous hermaphrodites. *Macrostomum lignano* is an outcrossing simultaneous hermaphrodite (Schärer & Ladurner, 2003) that copulates very frequently (Schärer et al., 2004). Copulations always occur reciprocally, with partners mutually inserting their male copulatory organ into the female genital pore (Schärer et al., 2004). Furthermore, it has been shown for this species that testis size is phenotypically plastic in response to different social group sizes, which is thought to be related to the average number of mates and therefore sperm competition intensity (Schärer & Ladurner, 2003). These findings indicate a high potential for sexual selection in *M. lignano* that is presumably mainly driven by post-copulatory mechanisms, i.e. sperm competition and cryptic female choice (*sensu* Thornhill, 1983).

In this study we experimentally tested how social group size and density affect the number of mates and sperm-transfer success in *M. lignano*. Although very little is known about correlates of multiple mating in hermaphrodites, we hypothesized that the number of mates increases with increasing social group size but that it would be unaffected by density as worms do not change their sex allocation when exposed to different densities (Schärer & Ladurner, 2003). Furthermore, we expected that worms transfer in total more sperm in larger social groups because this would explain why individuals enlarge their testes with increasing social group size, which has been shown several times for *M. lignano* (Schärer & Ladurner, 2003; Brauer et al., 2007; Schärer & Vizoso, 2007). Additionally, we tested whether morphological traits do explain variation in the number of mates and sperm-transfer success. Here, we focused only on traits that are potentially sexually selected. In particular, the analysis included body size, which has been shown to be involved in precopulatory mating decisions in other hermaphrodites by serving as a cue for fecundity of the mate (Vreys & Michiels, 1997; Anthes et al., 2006; but see Peters & Michiels, 1996; Koene et al., 2007). Furthermore, we tested the effect of ovary size and testis size, which have been demonstrated to reflect gonad productivity in *M. lignano* (Schärer & Vizoso, 2007; P. Sandner, unpublished data). As worms enlarge their testes in larger social groups (e.g. Schärer & Ladurner, 2003), we expect that individuals with larger testes are better competitors. In addition, we measured the size of the seminal vesicle, which has been shown to be highly correlated with the number of sperm that are available for subsequent matings (Schärer & Vizoso, 2007). Finally, we also studied the role of the male genital morphology, which has been demonstrated to be important for

fertilization success in gonochorists (e.g. House & Simmons, 2003).

Methods

Study organism

Macrostomum lignano (Macrostomorpha, Platyhelminthes) is a free-living flatworm of the intertidal meiofauna of the Northern Adriatic Sea (Ladurner *et al.*, 2005). In culture, it reaches 1.5 mm in body length and has a generation time of about 18 days. The worm is completely transparent allowing noninvasive measurement of internal morphology. The paired testes that occupy together about 6% of the body are located anterior to the paired ovaries (Ladurner *et al.*, 2005). The male genitalia include a false seminal vesicle, a muscular seminal vesicle, prostate gland cells, a vesicula granulorum and a sclerotized stylet (Fig. 1a). During copulation the stylet is inserted into the female genital pore of the mating partner, which opens into the female antrum where the sperm is stored.

In mass cultures, worms are maintained at 20 °C in glass Petri dishes containing f/2 medium and fed the diatom *Nitzschia curvilineata* (Andersen *et al.*, 2005). Worms used for this experiment originate from cultures of *M. lignano* that were initiated with individuals collected in 2003 near Lignano Sabbiadoro, Italy, by L. Schärer and D. B. Vizoso.

Morphometry

Morphological traits of focal worms were measured *in vivo* in a standardized way as described elsewhere (Schärer & Ladurner, 2003). We observed worms with a Leica DM 2500 microscope (Leica Microsystems, Wetzlar, Germany) and took digital photos at 40 × for body size and 400 × for gonad size, seminal vesicle size and stylet morphometry with a digital video camera (Sony DFW-X700; Sony Broadcast & Professional, Köln, Germany). Image acquisition was made using the software BTV Pro 6.0b1 (available at http://www.bensoftware.com/) and pictures were analysed with the image analysis software ImageJ 1.38× (available at http://rsb.info.nih.gov/ij/).

Morphometry of the stylet was analysed using the geometric morphometrics approach (Zelditch *et al.*, 2004). This landmark-based method allows quantifying the variation in stylet shape and size while removing information on position and orientation of the object. In total, we defined 24 landmarks that were superimposed for each stylet image (Fig. 1b) using tpsDig 2.10 (F. James Rohlf, 2006, Department of Ecology and Evolution, SUNY at Stony Brook; available at http://life.bio.sunysb.edu/morph/). Stylets of *M. lignano* typically exhibit a curvature that can be either orientated towards the false seminal vesicle or away from it whereas all intermediate shapes exist. By convention, we always
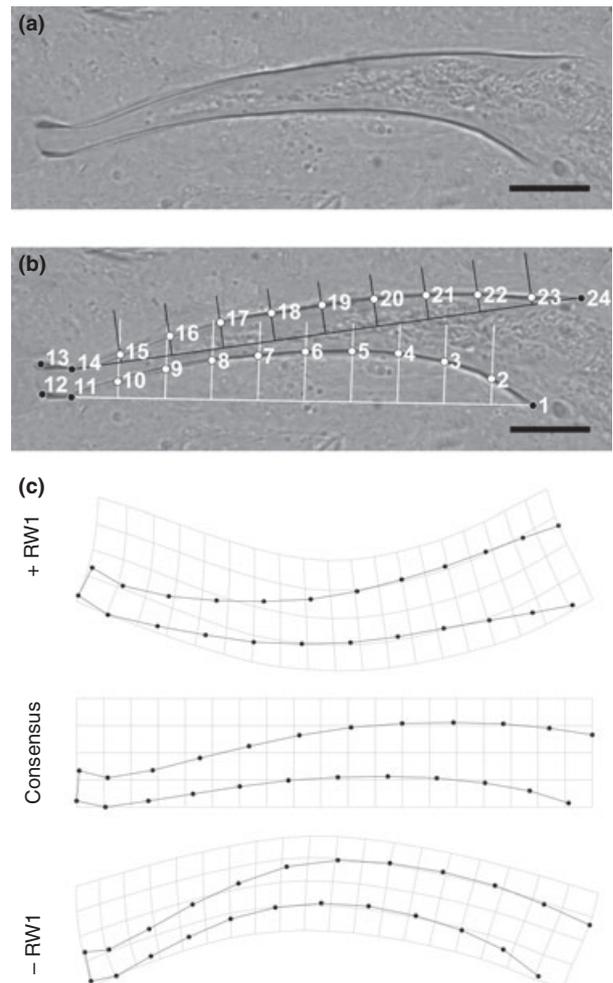
Fig. 1 Morphology and geometric morphometrics of the stylet of *Macrostomum lignano*. (a) Interference contrast micrograph of a typically shaped stylet. (b) Placement of fixed landmarks (black circles) and semi-landmarks (white circles). Numbers indicate the notation and the order in which all landmarks were placed. Standardized placement of semi-landmarks was achieved by drawing combs with equally spaced perpendicular lines between fixed landmarks (from 1 to 11 for white comb; from 14 to 24 for black comb) on each stylet image. (c) Thin-plate splines of the stylet derived from the relative warp score analysis. Panels show changes in the shape along first relative warp score. The consensus shape (middle) represents the average stylet shape. Stylets with high positive values of the first relative warp score are curved away from the seminal vesicle (above the consensus) whereas negative values refer to stylets that are curved strongly towards the seminal vesicle (below the consensus). For all stylets shown the seminal vesicle would be located below the stylet. The scale bars represent 10 μm.

placed the first landmark at the side of the base that was orientated towards the false seminal vesicle (Fig. 1b). Landmarks with the same notation should represent homologous points on the different stylets. Therefore, we could only define six fixed landmarks, including two at

the base and four at the tip of the stylet (Fig. 1b). All other points were located along the two curved sides of the stylet and were classified as so-called semi-landmarks (Zelditch *et al.*, 2004). To make these semi-landmarks comparable between individual stylets we followed the suggestions by Zelditch *et al.* (2004) and drew two combs with equally spaced perpendicular lines between fixed landmarks on all stylet images using the public domain software MakeFan 6 (H. David Sheets, 2003, available at http://www3.canisius.edu/~sheets/morphsoft.html), prior to superimposition. Semi-landmarks were then arranged at the intersection points of these lines with the stylet (Fig. 1b). We used the minimal bending energy method to slide the semi-landmarks along the outline curves using tpsRelw 1.45 (also available at http://life.bio.sunysb.edu/morph/). We used the same program for relative warp analysis. Relative warps are principal components of a distribution of shapes. Each relative warp score describes the direction of a particular shape change from the consensus form. The relative warp analysis yielded 44 relative warp scores from which the first three explained 83.0% of all variance in the shape of the stylet. The first relative warp score, which explained 57.7% of the variance in shape, described the most drastic change in stylet shape, namely the extent of the stylet curvature (Fig. 1c) and was used as the only stylet shape variable in our final models. Based on landmark data, we also extracted the centroid size of the stylet, which is the square root of the sum of squared distances between landmarks to their common centroid (Zelditch *et al.*, 2004) and a good estimator of overall stylet size.

A previous study has shown that measurements of body size, testis size, ovary size and seminal vesicle size are highly repeatable (Schärer & Ladurner, 2003). In order to verify the repeatability of our measurements of male copulatory organ shape and size we randomly selected 50 individuals from the mass cultures and conducted two complete runs of morphological measurements as outlined above for each single worm. Between the two runs, worms were allowed to relax for 12.9 ± 4.7 min (mean $\pm$ SD) in f/2 medium. The analysis of these repeated measurements confirmed that our measurements for stylet shape and size are repeatable (intraclass correlation coefficient r_i: first relative warp score, $r_i = 0.60$, $F_{49,50} = 3.9$, $P < 0.001$; centroid size, $r_i = 0.97$, $F_{49,50} = 73.4$, $P < 0.001$).

Sperm tracking

Sperm tracking was based on DNA-labelling of focal worms with a halogenated pyrimidine and the localization of the label using immunocytochemical staining. Worms were exposed to 5-bromo-2′-deoxyuridine (hereafter called BrdU), which is incorporated instead of thymidine into cells that undergo DNA replication (i.e. cells in s-phase). Thereby, spermatozoa of focal worms become labelled with BrdU when these cells

differentiate into sperm during spermatogenesis. Using this approach allows tracking the sperm of a labelled donor (hereafter called focal worms) in an unlabelled recipient.

The protocol was adapted from the method described in detail elsewhere (Schärer *et al.*, 2007). Focal worms were labelled by incubation in a solution of 0.5 mm BrdU (B5002-16; Sigma, St. Louis, MO, USA) in f/2 medium. A previous study showed that spermatogenesis in *M. lignano* takes about 6 days (Schärer *et al.*, 2007). In our study focal worms were continuously exposed to BrdU for 9 days, in order to assure that all sperm in the seminal vesicle of focal worms were labelled. Afterwards, focal and unlabelled worms were washed three times in artificial sea water before they were assigned to their experimental treatments. Fixation and immunocytochemical staining was carried out in tissue-culture plates while worms remained in their original social groups. After the mating trials (described below), worms were relaxed in a 5 : 3 mixture of 7.14% $MgCl_2$ and f/2 for 25 min and then fixated for 60 min in 4% paraformaldehyde in 0.1 m PBS with 10% sucrose. Fixated worms were washed three times with PBS-T (i.e. PBS plus 0.5% Triton X-100), followed by an additional 60 min wash with PBS-T and then permeated with 0.15 μg mL^{-1} Protease XIV at 37 °C for 35 min. Protease activity was stopped with cooled 0.1 n HCl. Subsequently, animals were transferred to 2 n HCl for 1 h at 37 °C, then washed three times with PBS-T and blocked with BSA-T (i.e. PBS-T plus 1% bovine serum albumin) for 60 min. BrdU-labelled cells were localized using a monoclonal rat anti-BrdU antibody (ab6326; Abcam Limited, Cambridge, UK) at a 1 : 100 dilution in BSA-T overnight at 4 °C. After four wash steps in PBS-T, the secondary goat-anti-rat FITC-conjugated antibody (ab6115; Abcam Limited) was applied in the dark for 1 h at room temperature at 1 : 200 in BSA-T. After three further wash steps in PBS-T, animals were mounted on microscope slides using Vectashield (Vector Laboratories, Burlingam, CA, USA), and stored at −20 °C until observation. BrdU-labelled sperm were visualized under epifluorescence on a Leica DM 5000 B microscope (Leica Microsystems). All sperm counts were performed blind with regard to the experimental treatment or the morphology of focal worms. Repeated examination of 50 randomly selected unlabelled sperm recipients confirmed a high repeatability of our sperm counts (intraclass correlation coefficient: $r_i = 0.99$, $F_{49,50} = 205.3$, $P < 0.001$).

Experimental set-up

On the first day we collected 1200 adult worms from mass cultures, distributed them equally among 12 glass Petri dishes and allowed them to lay eggs. After 72 h all worms were removed and the resulting hatchlings grew in f/2 medium until they had reached maturity (day 22),

yielding worms of similar age. We pooled all fully grown worms and randomly distributed 100 individuals to one Petri dish that was filled with a solution of 0.5 mM BrdU in f/2 medium (focal worms) and 100 worms to each of five Petri dishes filled with pure f/2 medium. Five days later we refreshed the BrdU solution and the f/2 medium. After a total of 9 days of BrdU exposure we photographed focal worms for morphological measurements and allowed them to recover overnight in isolation. Until the mating trials, all worms were kept under *ad libitum* food conditions (i.e. with a dense layer of diatoms on the bottom of the wells).

On day 32 we conducted the mating experiment. We manipulated social group size by forming social groups of two, three, four, eight and 16 individuals. Social groups were composed of one randomly selected focal worm and filled up with the respective number of unlabelled worms. Prior to the assignment of unlabelled worms into their final social group, they were pooled in order to balance any possible differences between worms kept in different Petri dishes. Density was manipulated by replicating all social group sizes in two different enclosure sizes (small or large, i.e. 24-hole and six-hole tissue culture plates containing 1.5 and 6.0 mL of f/2 medium respectively). Focal worms were allowed to mate in their social group and density treatment for 24 h and afterwards all worms were fixated and stained as described before. Due to time constraints, the whole experiment was split into two blocks that were separated by 4 days. Each block comprised six replicates of all factor combinations. As blocking the experiment had no significant effect on any of the variables measured (*t*-tests and Mann–Whitney *U*-tests: all $P > 0.05$) it was ignored in the final analysis. Several focal worms were lost due to pipetting errors during antibody staining ($n = 9$) or excluded because of malformation of the gonads or the copulatory organ ($n = 7$). The final data set included 648 individuals in 104 replicates with each factor combination being replicated on average 10.4 ± 1.6 times (mean ± SD; range: 8–12).

Statistical analysis

We used General Linear Models (GLM) in order to explain variation in the number of mates and sperm-transfer success. The number of mates was defined as the number of unlabelled individuals within a social group that had at least one labelled sperm in the female sperm storage organ. Therefore, the actual number of mates may be underestimated in this study because it is possible that there were some worms that mated but did not transfer any sperm (in case of nonreciprocal copulations) or did not succeed in storing any sperm in the sperm storage organ of their mate (due to sperm displacement or cryptic female choice). However, as we were more interested in explaining variation in the number of mates and less in reporting absolute values we expect that our

conclusions are robust despite this underestimation. Sperm-transfer success was described by two variables, namely the mean sperm-transfer success and total sperm-transfer success. Mean sperm-transfer success refers to the average number of labelled sperm the donor managed to get into storage per mate whereas total sperm-transfer success represents the sum of such sperm in a given social group. Measurements of sperm-transfer success presumably reflect the sperm allocation of a focal worm but also the outcome of sperm competition and cryptic-female choice.

For all three response variables we calculated GLMs with density and social group size as fixed factors and included body size, testis size, ovary size, seminal vesicle size, first relative warp score of the stylet and centroid size of the stylet as covariates. As body size correlated with testis size, ovary size and seminal vesicle size (Pearson correlation: testis size: $r = 0.56$, d.f. = 102, $P < 0.001$; ovary size: $r = 0.61$, d.f. = 102, $P < 0.001$; seminal vesicle size: $r = 0.20$, d.f. = 102, $P = 0.037$), we used the residuals of these traits derived from a linear regression fit against body size.

In simultaneous hermaphrodites, some authors combine estimates of male and female reproductive investment into a single variable to assess whether an individual allocates relatively more reproductive resources into one sex function in comparison with other individuals (e.g. Lorenzi *et al.*, 2005; Vizoso & Schärer, 2007). In a separate run of all models, we included such a composite measure of sex allocation as a covariate instead of testis size and ovary size. Sex allocation was defined as testes size of an individual divided by the total size of the gonads (ovaries and testes). Therefore, high values of sex allocation refer to more male-biased individuals. In this study, sex allocation of focal worms correlated positively with residual testis size ($r = 0.71$, d.f. = 102, $P < 0.001$) and negatively with residual ovary size ($r = -0.52$, d.f. = 102, $P < 0.001$). The models that included sex allocation did explain slightly less variation in our target variables compared with models testing gonad sizes separately. Furthermore, as the size of the male and female gonads were highly correlated with sex allocation, we only report statistics with residual testis size and residual ovary size as covariates in the results. For models including sex allocation as a covariate see Table S1 in the online Supporting Information.

In the models with the mean and the total sperm-transfer success as response variables we excluded focal worms whose number of mates was zero. This did not change any of the results reported qualitatively. Values of mean sperm-transfer success were log-transformed and total sperm-transfer success was square rooted. Residuals of all reported models did not deviate significantly from a normal distribution (Kolmogorov–Smirnov: all $P > 0.05$). Values are given as mean ± SE, unless otherwise stated. All statistics were carried out using SPSS 13.0 (SPSS Inc.,

Chicago, IL, USA) or JMP 7.0.1. (SAS Institute Inc., Cary, NC, USA).

Results

Morphological measurements

As intended by our random assignment of focal worms to the various treatments, there was no difference in morphological traits between social groups or densities (two-way ANOVA: all $P > 0.05$), except in centroid size of the stylet, which differed between social groups $(F_{4,94} = 2.9, P = 0.026)$. Moreover, none of the morphological traits that entered the final models correlated with each other (Pearson correlation: d.f. = 102, all $P > 0.05$), except centroid size of the stylet, which covaried negatively with residual testis size $(r = -0.22,$ d.f. = 102, $P = 0.023)$. Despite this collinearity between residual testis size and centroid size of the stylet we left both covariates together in each model because centroid size of the stylet did not explain any variation in the response variables when included instead or in combination with residual testis size. Nevertheless, because of the difference in centroid size between social groups the results regarding centroid size have to be considered with some caution.

Number of mates

The number of mates varied considerably in all social group sizes, ranging from zero to the maximum number of mates possible, except in social groups of 16 individuals where focal worms mated at most with 10 individuals. Out of 104 focal individuals 11 (10.6%) could not get any sperm into storage in any of the potential mates. The number of mates was significantly affected by social group size and residual testis size (Table 1). With increasing social group size the number of mates increased (mean number of mates, pairs: 0.9 ± 0.1; trios: 1.5 ± 0.7; quartets: 2.2 ± 0.9; octets: 2.8 ± 0.5; groups of 16 individuals: 5.4 ± 0.7; Fig. 2a,b). Furthermore, the residual testis size had a positive effect on the number of mates (Fig. 2b). The density and all other morphological traits that we measured did not significantly explain the remaining variance in the number of mates (Table 1).

Mean sperm-transfer success

Focal worms had a mean sperm-transfer success of 6.4 ± 0.7 sperm (range: 1–38 sperm). Mean sperm-transfer success was affected by social group size, residual testis size, residual ovary size and the first relative warp score of the stylet (Table 1). Worms that were kept in

Response	Source	d.f.	Std β	F-value	P-value
Number of mates*	Density	1	–	2.09	0.152
	Social group size	4	–	23.74	**< 0.001**
	Density × social group size	4	–	0.74	0.567
	Body size	1	0.01	0.01	0.968
	Residual testis size	1	0.26	10.88	**0.001**
	Residual ovary size	1	−0.01	0.02	0.900
	Residual seminal vesicle size	1	0.03	0.20	0.658
	First relative warp score	1	0.06	0.61	0.435
	Centroid size of stylet	1	0.08	1.05	0.309
Mean sperm-transfer success†	Density	1	–	0.51	0.474
	Social group size	4	–	10.82	**< 0.001**
	Density × social group size	4	–	0.08	0.987
	Body size	1	0.12	1.89	0.174
	Residual testis size	1	0.21	5.20	**0.025**
	Residual ovary size	1	−0.18	3.97	**0.049**
	Residual seminal vesicle size	1	0.13	2.10	0.151
	First relative warp score	1	0.27	7.54	**0.008**
	Centroid size of stylet	1	−0.02	0.04	0.839
Total sperm-transfer success‡	Density	1	–	0.03	0.870
	Social group size	4	–	0.80	0.529
	Density × social group size	4	–	0.41	0.800
	Body size	1	0.10	1.08	0.302
	Residual testis size	1	0.34	10.39	**0.002**
	Residual ovary size	1	−0.14	1.74	0.191
	Residual seminal vesicle size	1	0.13	1.65	0.203
	First relative warp score	1	0.32	8.32	**0.005**
	Centroid size of stylet	1	−0.07	0.46	0.500

Table 1 Results of General Linear Models performed to explain variation in the number of mates, mean sperm-transfer success (mean number of labelled sperm stored per mate) and total sperm-transfer success (total number of labelled sperm stored within a social group). Standardized beta (Std β) is the parameter estimate scaled to be dimensionless and indicates the relative importance of each covariate in explaining variation in the response variable (negative values indicate negative effects). Significant P-values are indicated in bold.

*Full model: $R^2 = 0.55$; $F_{15,88} = 7.05$; $P < 0.001$.
†Full model: $R^2 = 0.47$; $F_{15,77} = 4.55$; $P < 0.001$.
‡Full model: $R^2 = 0.29$; $F_{15,77} = 2.06$; $P = 0.021$.
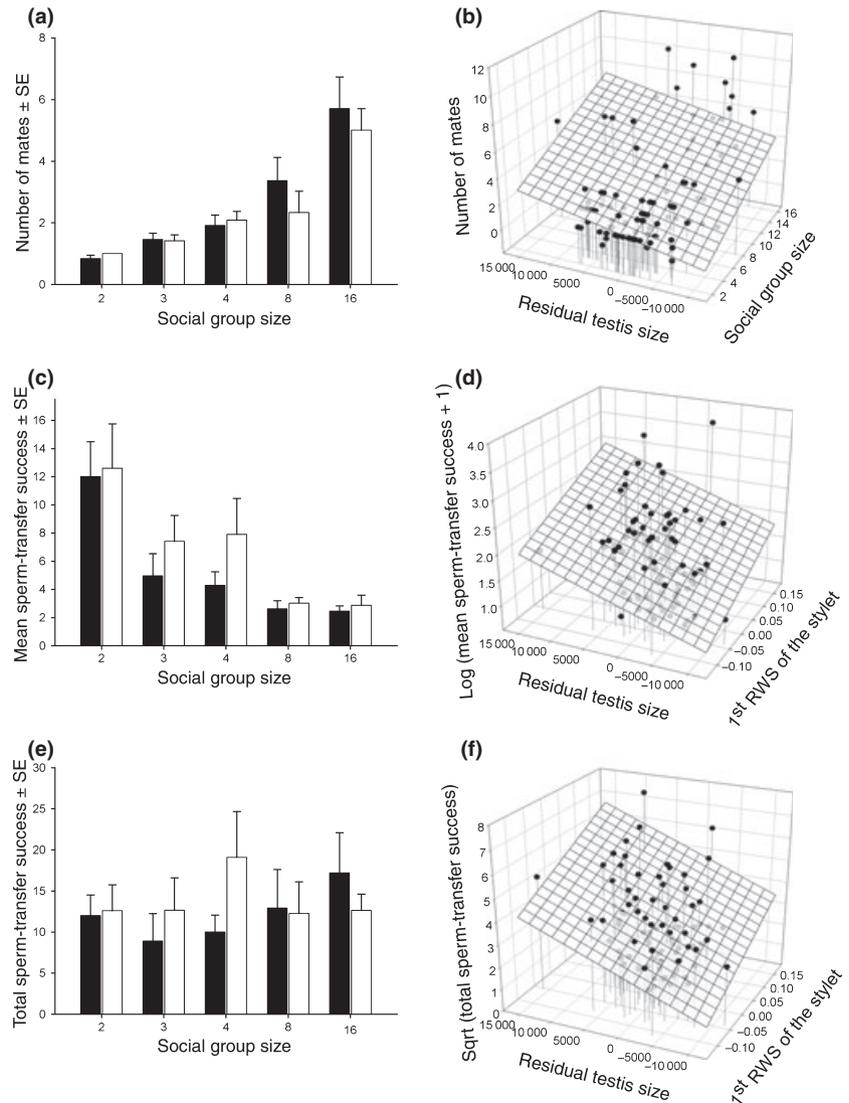
Fig. 2 Effects of social group size, density and morphology of sperm donors on the number of mates (a, b), mean sperm-transfer success (c, d) and total sperm-transfer success (e, f). Social group size includes the labelled focal worm. In the bar plots filled and open bars refer to the high- and low density treatment respectively. The plane grids in the three-dimensional scatter plots represent linear regression fits of both explanatory variables. First RWS refers to the first relative warp score obtained from the relative warp score analysis of the stylet. Note that response variables in bar plots are given with untransformed values. For statistics see Table 1.

larger social groups had a lower mean sperm-transfer success (Fig. 2c). Moreover, worms that had larger testes, smaller ovaries and stylets with high values of the first relative warp score (i.e. stylets that are curved away from the false seminal vesicle) had a higher mean sperm-transfer success (Fig. 2d). Density and all other morphological traits had no effect on mean sperm-transfer success (Table 1).

Total sperm-transfer success

Total sperm-transfer success was on average 13.3 ± 1.3 sperm (range: 1–61 sperm). There was no overall difference in total sperm-transfer success between social groups (one-way ANOVA: $F_{4,93} = 0.40$, $P = 0.809$). According to the full model, the total sperm-transfer success was only affected by residual testis size and the first relative warp score of the stylet (Table 1). Worms

with larger testes and with stylets that were curved away from the false seminal vesicle had an increased total sperm-transfer success (Fig. 2f). Density and social group size did not predict total sperm-transfer success (Table 1; Fig. 2e).

Discussion

This study focused on the degree of multiple mating and the causes of variation in the number of mates and sperm-transfer success in the simultaneously hermaphroditic flatworm *M. lignano*. We demonstrated that the number of mating partners is highly variable between individuals and showed that it is positively related to the social group size a worm is exposed to. In most of the social groups some worms managed to mate with all potential mating partners (except in groups of 16 individuals). This high degree of multiple mating violates the

central prediction of sex allocation theory that simultaneous hermaphroditism should only occur when the average number of mates is small (Charnov, 1982). High numbers of mating partners represent cases of high sperm competition intensity and therefore result in a lowered local mate competition in terms of competition between related sperm. However, local mate competition is assumed to be responsible for a saturating male fitness gain curve, which is required for hermaphroditism to be an evolutionarily stable strategy (Charnov, 1979, 1982). As our measure of the number of mates is potentially an underestimation of the actual number of mating partners (see Methods), this discrepancy between the number of mates in *M. lignano* and sex allocation theory for simultaneous hermaphrodites may even be more severe. The maximum number of mates in social groups of 16 individuals was 10, which suggests that there is an upper threshold of multiple mating in this species. However, this may also be caused by the relatively short period of only 24 h in which the worms were allowed to mate in our experiment.

We are aware of only one study that also assessed the number of mates in a simultaneously hermaphroditic animal. For the planarian flatworm *Schmidtea polychroa* it has been shown that adults produce offspring from an average of four mates when kept in groups of 10 individuals for 4 weeks (Pongratz & Michiels, 2003), which also indicates a fairly high degree of multiple mating. However, in this study social group size was not manipulated and analysis of paternity and maternity always ignores matings that failed to produce offspring (e.g. due to post-copulatory sexual selection or low hatching success), which would likely underestimate multiple mating more than is the case in our data. However, as both studies assessed multiple mating under laboratory conditions, there is a clear need for data on multiple mating in the field, in order to evaluate whether it is equally high under more natural conditions. No such data seem currently available.

Studies on sex allocation in hermaphrodites often manipulate social group size to produce situations with different levels of sperm competition, simply assuming that both are closely linked without explicitly testing this assumption (e.g. Trouvé *et al.*, 1999; Schärer & Ladurner, 2003; Tan *et al.*, 2004; Lorenzi *et al.*, 2005). Our study provides evidence that an increase in social group size indeed results in a higher average number of mates and therefore in a higher sperm competition intensity. In contrast, despite a fourfold increase in density, this factor had no statistically significant effect on the number of mates. Assuming an increased encounter rate at higher densities, this suggests that a higher encounter rate does not automatically lead to a higher degree of multiple mating. Alternatively, worms may gather in groups and may therefore not be randomly distributed in space. Consequently, individuals would not use all the available space, which would make them unaffected by enclosure

size. The lack of an effect of density agrees with earlier findings showing that *M. lignano* adjusts its sex allocation irrespective of density (Schärer & Ladurner, 2003; but see Schärer *et al.*, 2005).

In hermaphrodites, advantages in precopulatory mate competition and mate choice decisions have often been attributed to body size, with larger individuals being preferred mating partners because body size is often correlated with fecundity (for a review, see Leonard, 2006). In this study we found no relationship between body size and the number of mates. Hence, in *M. lignano* larger individuals are not capable of acquiring more mating partners. However, as we have no information on the body size of the unlabelled mates, we cannot rule out that there is size-assortative mating, which has previously been documented for other simultaneous hermaphrodites (Vreys & Michiels, 1997; but see Peters & Michiels, 1996; Michiels *et al.*, 2001; Koene *et al.*, 2007).

Interestingly, we found a positive effect of residual testis size on the number of mates. There are at least two possible explanations for this finding. First, as testis size reflects sperm production rate in *M. lignano* (Schärer & Vizoso, 2007), one could expect that individuals with larger testes are able to transfer sperm more often and/or in higher numbers per copulation. As our estimate of the number of mates is based on the occurrence of labelled sperm that is stored by mating partners and may therefore be subject to post-copulatory sexual selection, it is possible that higher numbers of transferred sperm could also lead to higher numbers of mating partners. However, the size of the seminal vesicle, which is a good estimate of the sperm reserves that are available for mating in *M. lignano* (Schärer & Vizoso, 2007), had no effect on the number of mates. Moreover, testis size of focal worms did not covary with the size of the seminal vesicle. Consequently, it seems unlikely that individuals with larger testes had more mating partners simply because they were able to transfer more sperm. However, this explanation cannot be ruled out because morphological measurements were carried out 1 day before the mating trials started and focal worms were kept in isolation during this time. Thus, focal worms could refill their sperm reserves during this isolation period, which may have rendered our measurement of seminal vesicle size as an estimate of sperm reserves unreliable.

As a second reason for the detected effect of testis size on the number of mates, we suspect that individuals varying in testis size behave differently. Sex allocation was highly correlated with testis size implying that worms with larger testes were also more male biased. Furthermore, an additional analysis including sex allocation as a composite measure of the relative allocation towards the male function confirmed that more male-biased individuals had more mates (see Supporting Information). Following the logic of Bateman's principle (see Introduction) more male-biased individuals may gain more from multiple mating than more female-

biased worms. Therefore, individuals with larger testes may be more eager to copulate. As testis size is pheno-typically plastic in *M. lignano* (Schärer & Ladurner, 2003), it is possible to manipulate this trait in this system, which offers the opportunity to test the positive relationship between testis size and the number of mates found in this study experimentally.

Our study also revealed large variation in sperm-transfer success, which was partly explained by social group size, residual testis size, residual ovary size and genital morphology. We found that focal worms had a lower mean sperm-transfer success in larger social groups. On the one hand, this could mean that worms reduce their ejaculate expenditure in larger groups as a response to an increased immediate sperm competition intensity as predicted by the theory on optimal sperm allocation (Parker *et al.*, 1996; Engqvist & Reinhold, 2005). On the other hand, it could simply be a consequence of repeated matings with the same partner in small social groups, with a constant number of sperm transferred in each mating irrespective of social group size. Additionally, another hypothesis is that only a limited number of sperm can be stored in the female sperm storage organ and that excess sperm is actively removed or gets lost passively (e.g. during egg laying). With a greater number of competitors this would also lead to a decrease in mean sperm-transfer success. Based on our data we are unable to distinguish between these nonexclusive hypotheses.

Surprisingly, the total sperm-transfer success remained constant across social groups. This may suggest that worms spent in total about equal amounts of sperm during the mating trials regardless of the number of potential mates and the level of sperm competition they experienced. Furthermore, together with our finding on the mean sperm-transfer success this indicates that worms do not adjust their male reproductive effort in terms of sperm investment according to the Coolidge effect, which predicts that males invest less reproductive resources when re-mated with the same mating partner (Dewsbury, 1981). On the assumption that our measure of total sperm-transfer success is closely related to the total sperm investment in *M. lignano*, one would expect a higher total sperm-transfer success in large groups compared to pairs because in the latter case worms can only mate with the same partner and should therefore invest less reproductive resources. However, in our study we found no difference in the total sperm-transfer success between all social group sizes. Even in groups of 16 individuals, where the average number of mates was about five individuals, worms had an equal total sperm-transfer success as they had in pairs. If sperm-transfer success is positively linked with sperm allocation, these findings suggest that worms do not allocate sperm prudently with respect to the Coolidge effect.

Sperm-transfer success was also affected by residual testis size. Both the mean and the total sperm-transfer success were positively related to testis size. This provides the first hint of ultimate benefits of having larger testes in *M. lignano*. One crucial assumption of sex allocation theory for simultaneous hermaphrodites is that the fitness of the male function is positively related to the proportion of resources allocated to the male function, depicted by a saturating fitness gain curve (Charnov, 1982). However, to our knowledge this relationship has never been empirically demonstrated for internally fertilizing hermaphroditic animals yet (for sperm-casting species, see McCartney, 1997; Yund, 1998). Assuming that the number of mates and sperm-transfer success are closely linked with the fitness of the male function, our study provides evidence that an increased male allocation leads to a higher reproductive success through the male function. In *M. lignano*, the effect of residual testis size on sperm-transfer success may be due to a higher mating frequency of more male-biased individuals, which would automatically lead to a higher number of stored sperm in the sperm storage organ of their mates (see discussion above). Another reason could be that more male-biased worms are better sperm competitors compared with more female-biased individuals because of differences in sperm displacement abilities or ejaculate quality. Interestingly, residual ovary size was negatively related to mean sperm-transfer success. This indicates that a higher reproductive success of the male function can only be achieved at the cost of the female function, which is probably a consequence of a trade-off in resource allocation to the male and the female function, which has been demonstrated for *M. lignano* (Schärer *et al.*, 2005). So far, very little is known about the effect of sex allocation on mating frequency and male competitiveness in simultaneous hermaphrodites, which highlights the need for experimental studies testing this hypothesis.

Another morphological trait that explained variation in sperm-transfer success was the shape of the stylet. To our knowledge, this study provides the first empirical evidence that the morphology of the male copulatory organ is an important predictor of sperm-transfer success in a simultaneous hermaphrodite. However, the reason why individuals with stylets that are curved away from the seminal vesicle (indicated by positive scores of the first relative warp) have a higher sperm-transfer success remains unknown. One potential mechanism is that specifically shaped stylets prevent sperm from being subject to cryptic female choice, e.g. by modifying the shape of the sperm storage organ or by optimal positioning of sperm within the female genitalia. Alternatively, stylet shape may also be important to out-compete sperm inseminated by other worms via sperm displacement, which would be expected to generate second male sperm precedence. A detailed understanding of the processes during and after copulation with special emphasis on stylet intromission, sperm release, sperm displacement and accompanied changes in the conformation of the female sperm storage organ would help to identify the

underlying mechanisms that lead to an advantage of specifically shaped stylets in sperm-transfer success.

In conclusion, this study demonstrates that individuals of *M. lignano* differ considerably in the number of mates and sperm-transfer success. We reveal that social group size and morphological traits of the sperm donor are important factors to explain variation in these parameters in a simultaneous hermaphrodite. Our findings support the idea that more male-biased individuals are able to acquire more mating partners and have a higher sperm-transfer success. Additionally, we show for the first time in hermaphrodites that genital morphology is an important predictor of sperm-transfer success. Further studies should experimentally test how sex allocation affects mating behaviour and explain how stylet shape influences sperm-transfer success in *M. lignano* mechanistically.

Acknowledgments

References

Andersen, R.A., Berges, J.A., Harrison, P.J. & Watanabe, M.M. 2005. Recipes for freshwater and seawater media. In: *Algal Culturing Techniques* (R.A. Andersen, ed.), pp. 429–538. Elsevier, Amsterdam.

Anthes, N., Putz, A. & Michiels, N.K. 2006. Hermaphrodite sex role preferences: the role of partner body size, mating history and female fitness in the sea slug *Chelidonura sandrana*. *Behav. Ecol. Sociobiol.* **60**: 359–367.

Bateman, A.J. 1948. Intra-sexual selection in *Drosophila*. *Heredity* **2**: 349–368.

Brauer, V.S., Schärer, L. & Michiels, N.K. 2007. Phenotypically flexible sex allocation in a simultaneous hermaphrodite. *Evolution* **61**: 216–222.

Bussière, L.F., Hunt, J., Jennions, M.D. & Brooks, R. 2006. Sexual conflict and cryptic female choice in the black field cricket, *Teleogryllus commodus*. *Evolution* **60**: 792–800.

Charnov, E.L. 1979. Simultaneous hermaphroditism and sexual selection. *Proc. Natl Acad. Sci. USA* **76**: 2480–2484.

Charnov, E.L. 1982. *The Theory of Sex Allocation*. Princeton University Press, Princeton, NJ.

Dewsbury, D.A. 1981. Effects of novelty on copulatory behavior – the Coolidge effect and related phenomena. *Psychol. Bull.* **89**: 464–482.

Eberhard, W.G. 1996. *Female Control: Sexual Selection by Cryptic Female Choice*. Princeton University Press, Princeton, NJ.

Engqvist, L. & Reinhold, K. 2005. Pitfalls in experiments testing predictions from sperm competition theory. *J. Evol. Biol.* **18**: 116–123.

Fischer, E.A. 1981. Sexual allocation in a simultaneously hermaphroditic coral-reef fish. *Am. Nat.* **117**: 64–82.

Frank, S.A. 2002. A touchstone in the study of adaptation. *Evolution* **56**: 2561–2564.

Hamilton, W.D. 1967. Extraordinary sex ratios. *Science* **156**: 477–488.

House, C.M. & Simmons, L.W. 2003. Genital morphology and fertilization success in the dung beetle *Onthophagus taurus*: an example of sexually selected male genitalia. *Proc. R. Soc. Lond. B Biol. Sci.* **270**: 447–455.

Klinkhamer, P.G.L., deJong, T.J. & Metz, H. 1997. Sex and size in cosexual plants. *Trends Ecol. Evol.* **12**: 260–265.

Koene, J.M., Montagne-Wajer, K. & Ter Maat, A. 2007. Aspects of body size and mate choice in the simultaneously hermaphroditic pond snail *Lymnaea stagnalis*. *Anim. Biol.* **57**: 247–259.

Ladurner, P., Schärer, L., Salvenmoser, W. & Rieger, R.M. 2005. A new model organism among the lower Bilateria and the use of digital microscopy in taxonomy of meiobenthic Platyhelminthes: *Macrostomum lignano*, n. sp. (Rhabditophora, Macrostomorpha). *J. Zool. Syst. Evol. Res.* **43**: 114–126.

Leonard, J.L. 2006. Sexual selection: lessons from hermaphrodite mating systems. *Integr. Comp. Biol.* **46**: 349–367.

Lorenzi, M.C., Sella, G., Schleicherova, D. & Ramella, L. 2005. Outcrossing hermaphroditic polychaete worms adjust their sex allocation to social conditions. *J. Evol. Biol.* **18**: 1341–1347.

McCartney, M.A. 1997. Sex allocation and male fitness gain in a colonial, hermaphroditic marine invertebrate. *Evolution* **51**: 127–140.

Michiels, N.K., Hohner, A. & Vorndran, I.C. 2001. Precopulatory mate assessment in relation to body size in the earthworm *Lumbricus terrestris*: avoidance of dangerous liaisons? *Behav. Ecol.* **12**: 612–618.

Parker, G.A. 1970. Sperm competition and its evolutionary consequences in insects. *Biol. Rev.* **45**: 525–567.

Parker, G.A., Ball, M.A., Stockley, P. & Gage, M.J.G. 1996. Sperm competition games: individual assessment of sperm competition intensity by group spawners. *Proc. R. Soc. Lond. B Biol. Sci.* **263**: 1291–1297.

Peters, A. & Michiels, N.K. 1996. Do simultaneous hermaphrodites choose their mates? Effects of body size in a planarian flatworm. *Freshw. Biol.* **36**: 623–630.

Pizzari, T. & Birkhead, T.R. 2000. Female feral fowl eject sperm of subdominant males. *Nature* **405**: 787–789.

Pongratz, N. & Michiels, N.K. 2003. High multiple paternity and low last-male sperm precedence in a hermaphroditic planarian flatworm: consequences for reciprocity patterns. *Mol. Ecol.* **12**: 1425–1433.

Reece, S.E., Drew, D.R. & Gardner, A. 2008. Sex ratio adjustment and kin discrimination in malaria parasites. *Nature* **453**: 609–614.

Schärer, L. & Ladurner, P. 2003. Phenotypically plastic adjustment of sex allocation in a simultaneous hermaphrodite. *Proc. R. Soc. Lond. B Biol. Sci.* **270**: 935–941.

Schärer, L. & Vizoso, D.B. 2007. Phenotypic plasticity in sperm production rate: there's more to it than testis size. *Evol. Ecol.* **21**: 295–306.

Schärer, L., Joss, G. & Sandner, P. 2004. Mating behaviour of the marine turbellarian *Macrostomum* sp.: these worms suck. *Mar. Biol.* **145**: 373–380.

Schärer, L., Sandner, P. & Michiels, N.K. 2005. Trade-off between male and female allocation in the simultaneously

hermaphroditic flatworm *Macrostomum* sp. *J. Evol. Biol.* **18**: 396–404.

Schärer, L., Zaubzer, J., Salvenmoser, W., Seifarth, C. & Ladurner, P. 2007. Tracking sperm of a donor in a recipient: an immunocytochemical approach. *Anim. Biol.* **57**: 121–136.

Tan, G.N., Govedich, F.R. & Burd, M. 2004. Social group size, potential sperm competition and reproductive investment in a hermaphroditic leech, *Helobdella papillornata* (Euhirudinea: Glossiphoniidae). *J. Evol. Biol.* **17**: 575–580.

Thornhill, R. 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. *Am. Nat.* **122**: 765–788.

Trouvé, S., Jourdane, J., Renaud, F., Durand, P. & Morand, S. 1999. Adaptive sex allocation in a simultaneous hermaphrodite. *Evolution* **53**: 1599–1604.

Vizoso, D.B. & Schärer, L. 2007. Resource-dependent sex allocation in a simultaneous hermaphrodite. *J. Evol. Biol.* **20**: 1046–1055.

Vreys, C. & Michiels, N.K. 1997. Flatworms flatten to size up each other. *Proc. R. Soc. Lond, B Biol. Sci.* **264**: 1559–1564.

Yund, P.O. 1998. The effect of sperm competition on male gain curves in a colonial marine invertebrate. *Ecology* **79**: 328–339.

Zelditch, M.L., Swiderski, H.D., Sheets, H.D. & Fink, W.L. 2004. *Geometric Morphometrics for Biologists: A Primer*. Elsevier Academic Press, London.

Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 Results of General Linear Models performed to explain variation in the number of mates, mean sperm-transfer success and total sperm-transfer success.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.