

Tracking sperm of a donor in a recipient: an immunocytochemical approach

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Abstract—In order to study the mechanisms of sperm competition and cryptic female choice we require an understanding of the patterns of sperm storage, sperm removal and sperm digestion. Current studies infer these patterns mainly from paternity data, which only reveal the ultimate outcomes of the interactions between male and female reproductive processes. However, only with a mechanistic understanding of the fate of received sperm, and the involved patterns of postcopulatory sexual selection, can we understand the evolution of male and female reproductive morphology and physiology. The currently available approaches for tracking donor sperm in a recipient are either very time consuming, spatially imprecise or limited to model organisms for which considerable genetic knowledge and molecular know-how is available. Using the free-living flatworm *Macrostomum lignano* we here present a novel sperm tracking approach that uses DNA-labelling with a halogenated pyrimidine and localisation of the label using immunocytochemistry. We first outline modifications to established protocols to allow visualisation of gametic cells, in addition to somatic cells, determine the duration and patterns of spermatogenesis, and then show that labelled sperm from labelled donors can be observed in unlabelled recipients. We further show that labelled worms have a mating behaviour that is comparable to that of unlabelled worms except in one parameter. We suggest ways in which this approach can be optimised, and that it should be readily transferable to other taxa. We conclude that this approach will be a valuable tool to study postcopulatory sexual selection.

Keywords: BrdU, cryptic female choice; Platyhelminthes; postcopulatory sexual selection; sperm competition; sperm tracking, spermatogenesis.

INTRODUCTION

Historically, studies of sperm competition were restricted to the determination of the patterns of paternity resulting from double mating of a female, i.e., the

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determination of so-called P_2 -values (where P_2 = the proportion of offspring sired by the second male to mate; Parker, 1998). Initially, these patterns were inferred from phenotypically visible genetic markers with simple Mendelian inheritance (e.g., eye colour). However, these are not always selectively neutral and are often only available in well-established model organisms. Alternatively, researchers have used the sterile male technique (Parker, 1970) in which males are exposed to a dose of radiation high enough to produce failed development of eggs fertilised with irradiated spermatozoa, but low enough to prevent, at least in the short-term, damage to the physical performance of irradiated males and sperm (Toledo *et al.*, 2004). Unless this performance is comparable between irradiated and control treatments, P_2 -values can be misleading. Moreover, recent evidence has clearly shown that the sterile male technique needs to be used with care, particularly when temporal patterns of sperm competition are studied, because irradiated males can sometimes regain fertility (Rugman-Jones and Eady, 2001), possibly by recruiting new spermatogonia from radio-protected stem cells in the testis. Nevertheless, some studies have confirmed that, when used properly, the sterile male technique can give very similar results to genetic markers (Eady, 1991). More recently, DNA analyses, and particularly microsatellite marker analyses, have become the method of choice for paternity analyses (see discussion, Simmons and Achmann, 2000). However, the establishment of microsatellites is both laborious and costly, and may thus not be readily available for all study species.

Paternity patterns only allow inferences about the ultimate outcome of sperm competition, and not the study of the interaction of ejaculates from different males. For example, considerable interest has recently arisen for female-driven processes, which involve selective handling of sperm from different males, and which can result in cryptic female choice (see review, Birkhead and Pizzari, 2002). Such processes can be studied only indirectly using paternity analyses. Studies have therefore tried to measure the fate of ejaculates and sperm transferred by a sperm donor to a recipient, and have aimed at determining so-called S_2 -values (where S_2 = the proportion of sperm stored at a given sperm storage site by the second male to mate). The aim is to track the donor's ejaculate and/or sperm in the recipient. Studies of this type have used one of four approaches (although not all of these were actually aimed at estimating S_2 -values). The first involves unspecific labelling of the donor individual with radiolabelled amino acids (Simmons *et al.*, 1999). This leads to incorporation of radiolabel in all tissues, including the testis and accessory glands, and thus to the labelling of the entire ejaculate of sperm donors. The radiolabel can then be localised by scintillation counting of different tissues or by autoradiography. The second approach is to use radiolabelled nucleotides which lead to specific labelling of the DNA of the sperm donor (including the DNA of the donor's sperm). It generally uses tritiated thymidine (^3H -thymidine) which can be visualised by scintillation counting or autoradiography (Nollen, 1975, 1997; Bishop, 1996). However, both of these approaches have limitations. In the case of scintillation counting, the signal cannot be very precisely localised morphologically. In the case

of autoradiography, very accurate localisation of the label is possible, but it requires laborious serial sectioning of the tissues of interest to provide a sufficiently close association between the labelled tissue and the radiographic emulsion. Furthermore, exposure times are usually several weeks which makes this approach even more time consuming.

A third, and very elegant, approach to estimate S_2 -values is to use the well-documented fact that, in many organisms, the sperm produced by one individual are very similar in size, but that there is considerable variation in average sperm size between individuals of the same population (Morrow and Gage, 2001; Oppliger et al., 2003; Minoretta and Baur, 2006). By staging matings between individuals with non-overlapping sperm size ranges, the S_2 -values can be determined by assigning sperm in storage to their donor according to their size (Hellriegel and Bernasconi, 2000). A potential drawback of this approach, on top of the need to dissect out sperm to accurately determine their length, is that sperm size could, of course, itself influence the competitive ability of sperm.

Finally, a new approach to sperm tracking uses the construction of transgenic strains which express a fluorescent marker, usually green-fluorescent protein (GFP), in the sperm (Civetta, 1999). The sperm of transgenic individuals can thus be tracked simply by observing them *in situ* under epifluorescence or in a laser scanning microscope (particularly if organisms are transparent and can be immobilised sufficiently). GFP-like protein has, in recent years, been engineered to emit fluorescence of at least five different wavelengths (see <http://www.clontech.com/clontech/gfp/index.shtml>) which would allow tracking of sperm from several males simultaneously. A serious drawback of this approach is that it is currently only possible in well-established model organisms, such as *Drosophila*, because it not only requires detailed knowledge of which genes are expressed in sperm, but also knowledge of the promoters of these genes and methods for the production of transgenic strains. In addition, stable incorporation of the GFP-constructs into the germ-line can often be difficult, and the fitness effects of construct insertion may be substantial. All these drawbacks limit the utility of this approach, and it is thus, at least at the moment, beyond the reach for many organisms that are currently used to study sperm competition.

Here we present a novel approach which: i) allows the tracking of sperm of a sperm donor in a sperm recipient; ii) should be readily transferable to a wide range of organisms, and iii) does not require specific genetic knowledge. This approach uses DNA-labelling with a halogenated pyrimidine, the thymidine analogue 5-bromo-2'-deoxyuridine (hereafter called BrdU). We have previously used this approach in a study on male allocation (Schärer et al., 2004b). BrdU is stably incorporated into the DNA of cells in S-phase (i.e. the phase of the cell cycle during which DNA duplication occurs) and can be visualised with immunocytochemical staining. We describe a protocol for BrdU-based sperm tracking in the free-living marine flatworm *Macrostomum lignano* that allows the visualisation of labelled sperm from a labelled donor in an unlabelled recipient.

MATERIALS AND METHODS

Study animal

Macrostomum lignano (Macrostomorpha, Platyhelminthes) is a free-living flatworm from the interstitial sand fauna of the northern Adriatic Sea (Ladurner et al., 2000, 2005b). It is an obligately outcrossing, simultaneous hermaphrodite with reciprocal copulation, reaches 1.5 mm in length and has a generation time of only 18 days (Schärer and Ladurner, 2003; Schärer et al., 2004a). The paired testes are located anterior to the paired ovaries (fig. 1). Vasa deferentia emanate from each testis, which join each other shortly before entering a very flexible false seminal vesicle (essentially a distended common vas deferens). The bulk of sperm ready to be transferred to mating partners are stored in this false seminal vesicle, which in turn is connected to the muscular true seminal vesicle, which pumps the sperm into the copulatory stylet during copulation. Sperm are transferred into the female antrum (i.e. the sperm storage organ) of the sperm recipient.

Mass cultures of *Macrostomum lignano* are maintained in f/2, which is an artificial seawater medium (Andersen et al., 2005), in a climate room at 20°C and a 14:10 h light: dark cycle, and fed with the diatom, *Nitzschia curvilineata* (Tyler, 1981; Rieger et al., 1988).

General immunocytochemistry and label visualisation

The methods used here were modified from those described in Schärer et al., 2004 (see also Discussion). All labelling steps were done in polystyrene tissue culture plates or in small glass embryo dishes. Animals were labelled by incubation in 5 mM BrdU (5-bromo-2'-deoxyuridine, B5002, Sigma-Aldrich, Vienna, Austria) in f/2 medium, then thoroughly washed and kept as before for different lengths of time depending on the question under study (for details, see Experiments 1-3 below).

Before fixation animals were relaxed in a 5:3 mixture of 7.14% MgCl₂ and f/2 for 10-20 min and then fixed for 60 min in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) with 10% sucrose. Fixed worms were washed three times with PBS, followed by a 60 min wash with PBS-T (i.e. PBS plus 0.1% Triton X-100). They were permeated with 0.15 µg/ml Protease XIV at 37°C. Protease activity was visually checked and stopped with cooled 0.1 N HCl. Animals were then transferred first to 2 N HCl for 1 h at 37°C, then washed several times with PBS-T and blocked with BSA-T (i.e. PBS-T plus 1% bovine serum albumin) for 30 min. Cells that had incorporated BrdU were localised 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, Cambridge, UK) was applied 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), and stored at -20°C until observation.

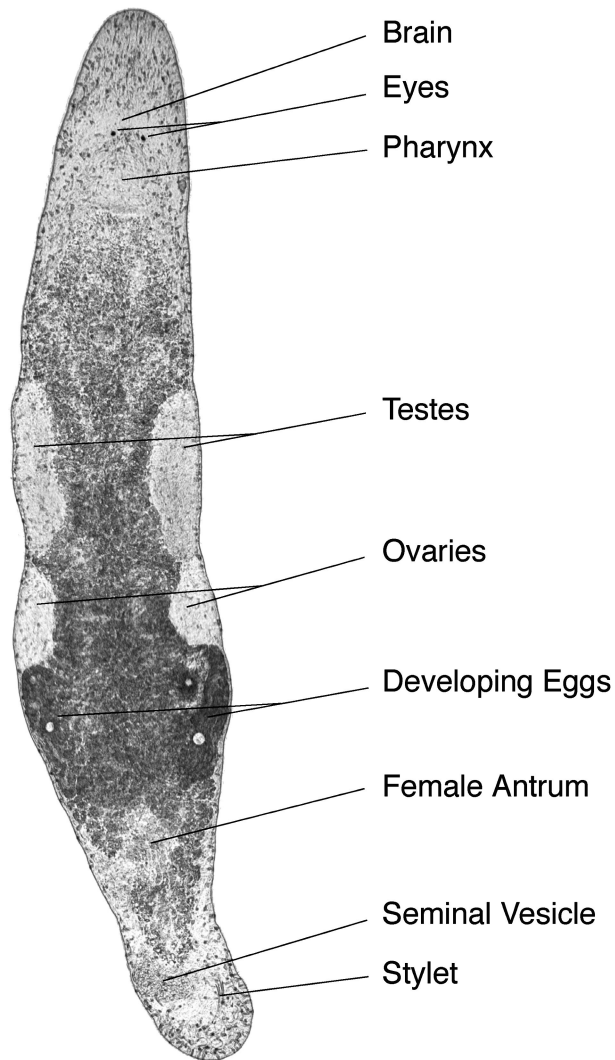


Figure 1. A living adult *Macrostomum lignano*, lightly squeezed between a microscope slide and a cover glass. Note that many internal structures and organs can be readily observed. (The length of the worm is about 1.5 mm.)

BrdU-positive cells were visualised either under epifluorescence on a Polyvar (Reichert, Austria) or a Leica DM 5000 B (Leica, Austria), or with a Zeiss LSM 510 confocal laser scanning microscope (Zeiss, Germany) with Plan Neofluoar 40x or 63x oil objectives. On the LSM we made z-stacks of up to 70 optical slices (each 512×512 pixels) 0.63 or $0.39 \mu\text{m}$ thick. Maximum intensity z-projections were done of a subset of the whole stack using ImageJ 1.35j (available at <http://rsb.info.nih.gov/ij/>).

Experiment 1: Duration of spermatogenesis

We determined the duration of spermatogenesis by following the fate of the incorporated BrdU label that we had administered in a 30 min pulse (i.e., we performed pulse-chase experiments). Over 6 days, six worms were pulsed each day around noon and then all worms were fixed and stained together on the final day. This produced worms in which the fate of the label was followed for 0, 24, 48, 71, 97, 121 or 145 h.

Experiment 2: Transfer and tracking of labelled sperm

Here 40 worms (further called labelled worms) were pulse-labelled with BrdU for 17 h and held under normal culture conditions for a chase-time of 6 days. Labelled worms were then transferred to a fresh algae dish together with 60 unlabelled worms and allowed to mate for 2 days. All 100 worms were fixed and processed for immunocytochemistry, observed under epifluorescence, and scored for whether they were labelled or unlabelled. For labelled worms, we further checked if they had labelled sperm in the seminal vesicle (i.e., if they were able to transfer labelled sperm), and for unlabelled worms we checked if they had labelled received sperm in their female antrum (i.e., if they had copulated with, and received sperm from, labelled worms).

Experiment 3: Mating behaviour of labelled worms

In order to check if the labelled worms have a normal copulation behaviour, we staged matings between a labelled and an unlabelled worm ($n = 45$, BrdU treatment) and matings between two unlabelled worms ($n = 45$, control treatment). On day 1 a subset of 45 worms of a batch of 180 adult worms were pulse-labelled with BrdU for 10 h. On days 4 or 5 (i.e. before labelled worms had labelled mature sperm, see results of Experiment 1) all 180 worms were isolated in 24-well tissue culture plates. On day 7 worms were randomly assigned to a mating partner with which they were allowed to mate for 30 min in a observation chamber while being filmed using a digital video camera setup (for details see Schärer et al., 2004a). Six pairs were filmed simultaneously in each observation chamber for a total of 15 observation chambers. In contrast to the method described earlier, the microscope slides for the observation chamber were siliconised with Sigmacote (SL2, Sigma-Aldrich, Vienna, Austria), according to the instructions of the manufacturer. This allowed us to open observation chambers after filming and to retain the pair identity of worms in pairs for further analysis, because it prevented drops from flowing into each other. After mating worms were immediately fixed and treated for immunocytochemistry. Two BrdU-treatment pairs had to be excluded due to pipetting errors, so the final sample size was $n = 43$ for the BrdU treatment and $n = 45$ for the control treatment. We scored if the pair mated and determined the number of copulations. We further determined the numbers of sucks that the worms

performed. Suck is a postcopulatory behaviour in which the worms bend down and place their pharynx over their own female genital opening, and then appear to perform a sucking behaviour. The function of this behaviour is currently unknown. Sucks can be performed by none, one (single suck) or both (double suck) worms of a pair after copulation (Schärer et al., 2004a).

Of the two unlabelled worms in each control treatment pair, only one was chosen at random to be analysed immunocytochemically. Further, 11 worms were lost during the staining procedure, yielding 39 labelled and 34 unlabelled worms for the BrdU treatment and 37 unlabelled worms for the control treatment. The scoring of the unlabelled worms was done blind with respect to the treatment group. This allowed us to check if the structures that we considered to be labelled sperm in the female antrum actually only occurred in the unlabelled worms from the BrdU treatment.

RESULTS

Experiment 1: Duration of spermatogenesis

Figure 2 shows representative stages of the spermatogenesis of *Macrostomum lignano*. BrdU incorporation into spermatogonia after 0 h was as previously reported by Schärer et al., 2004b (data not shown). After 24 h the nuclear structure of the labelled nuclei was somewhat less dense, and after 48 h nuclei appeared to become a little larger. At 71 h nuclei were more diffuse and some appeared to have meiotic figures, suggesting that the cells had reached the spermatocyte stage. At 97 h nuclei became more condensed and often occurred in groups of four cells. These nuclei belong to spermatids, which we have previously observed to occur in groups of four based on a monoclonal antibody (Ladurner et al., 2005a). At 121 h the nuclei had clearly started to elongate, but had not yet reached the full length, thus indicating the beginning of the spermiogenesis. These elongating nuclei also often appeared in groups of four. Together with the observation on the spermatids this would suggest that there are no mitotic steps after the spermatogonial division in the spermatogenesis of *M. lignano*. After 145 h some nuclei appeared to have reached their full length (nuclei of ripe sperm are about 25 μm long; L. Schärer, pers. obs.), and in some worms labelled sperm were already observed in the seminal vesicle, suggesting that 6 days are sufficient to complete spermatogenesis.

Experiment 2: Transfer and tracking of labelled sperm

Of the 100 worms that we used for this experiment, 36 were lost in the staining process. This can occur if many worms are stained together as they get more easily damaged or stuck in the pipette. Of the remaining 64 worms, 22 were labelled and 42 were unlabelled (fig. 3). In 21 of the 22 labelled worms we could observe

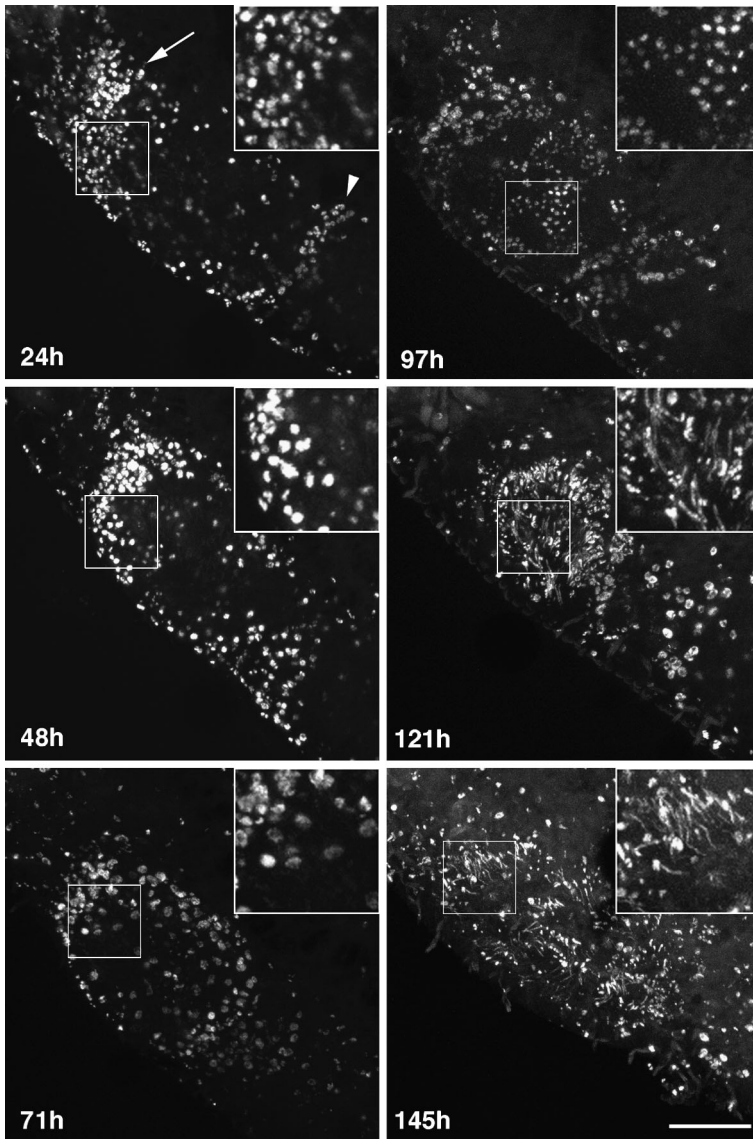


Figure 2. Duration and stages of spermatogenesis in *Macrostomum lignano*. The panels show one testis each at different times after a 30' BrdU pulse. The upper left corner of the panels is anterior, the denser cluster of BrdU-positive cells is the stem cell cluster of the testis (arrow), and the less dense, posterior cluster of BrdU-positive cells is the stem cell cluster of the ovary (arrowhead). The small white squares indicate the regions that are displayed in the insets at 2x magnification. The figures are z-projections of confocal stacks. See Results for details on stages. (Scale bar is 50 μm for all panels.)

labelled sperm in the seminal vesicle (fig. 4), suggesting that the labelling technique and the timing of the experiment are appropriate. Of the 42 unlabelled worms, 33 had received labelled sperm in the female antrum (fig. 5). The number of labelled

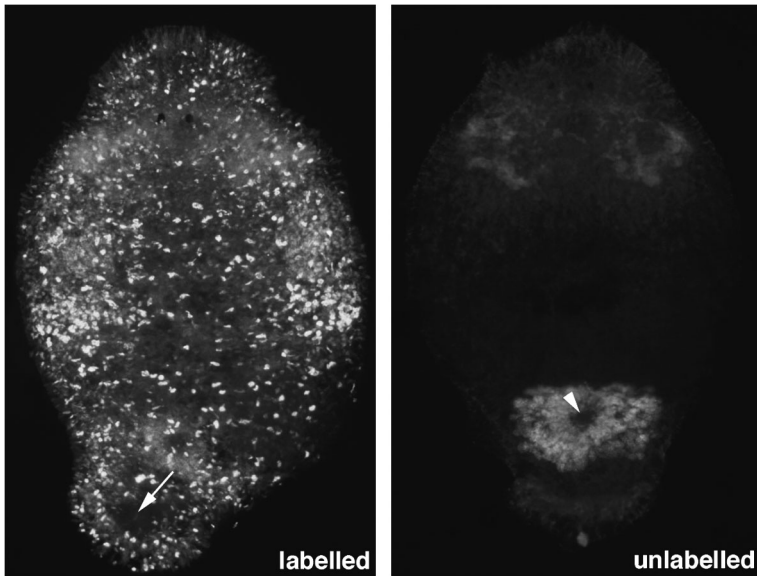


Figure 3. A labelled and an unlabelled *Macrostomum lignano*, both of which were stained immunocytochemically to visualise incorporated BrdU. Note the contrast between the many labelled cells in the labelled worm (the arrow indicates the location of the seminal vesicle), and the absence of labelled cells in the unlabelled worm (the arrowhead indicates the location of the vagina and the female antrum; note that no received sperm can be seen at this magnification). The vagina is surrounded by shell gland granules which are involved in the formation of the egg shell during egg laying, and which are stained in an unspecific way by the secondary antibody. Unspecific labelling of these granules is a general feature of a number of secondary antibodies, and can be readily distinguished from labelled sperm both in shape and staining intensity.

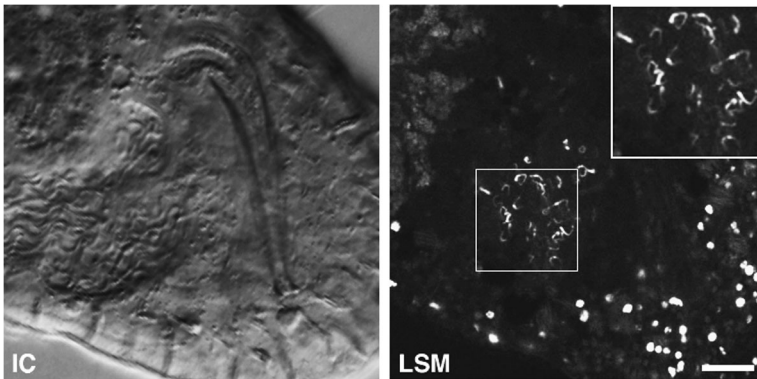


Figure 4. The seminal vesicle and copulatory stylet of *Macrostomum lignano*. The left panel shows an interference contrast (IC) picture of the tail region of a living worm. Mature sperm can be observed in the seminal vesicle. The right panel shows a z-projection of a confocal stack (LSM) of a BrdU-labelled worm. Labelled sperm nuclei can be observed in the seminal vesicle. The small white square indicates the region that is displayed in the insets at 1.5x magnification. (Scale bar for the LSM panel is 20 μm .)

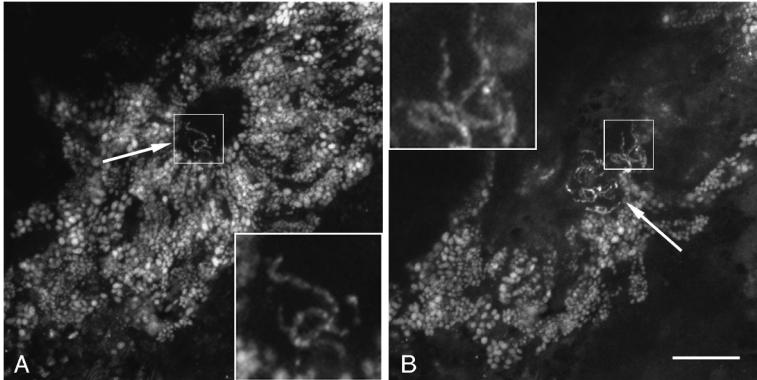


Figure 5. Labelled received sperm in the female antrum of an unlabelled *Macrostomum lignano*. The two panels show optical slices at different levels of a confocal stack through the female antrum. Panel A shows the unspecific staining of shell gland granules around the vagina in which 2-3 sperm are visible (arrow). Panel B shows the lumen of the female antrum which contains considerable amounts of sperm (arrow). The small white squares indicate the regions that are displayed in the insets at 3x magnification. (Scale bar is 20 μm for both panels.)

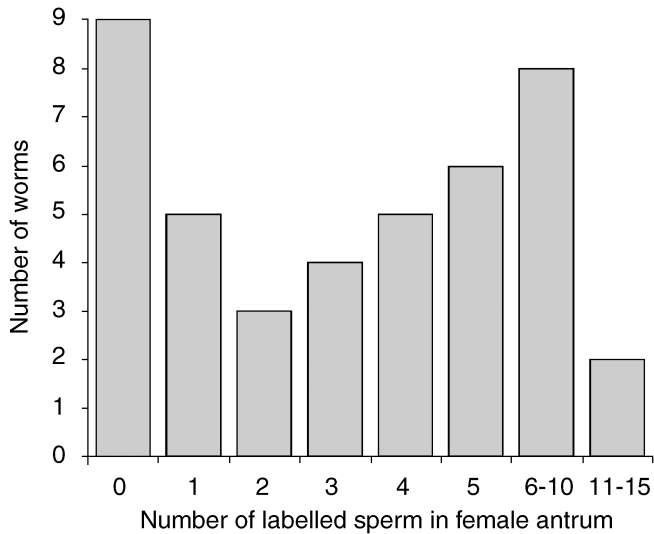


Figure 6. Transfer of BrdU-labelled sperm in *Macrostomum lignano*. The figure shows the frequency distribution of the number of labelled sperm in the female antrum of unlabelled worms.

received sperm ranged from one to approximately 15 (fig. 6), suggesting that labelled worms were capable of mating and able to transfer sperm to their mating partners. When few of the received sperm were labelled they could be counted individually, but, due to the long and often twisted sperm nuclei, it can be difficult to count the sperm when they are numerous.

Experiment 3: Mating behaviour of labelled worms

There was no significant difference between both treatments in the number of pairs that failed to mate in the 30 min observation period (BrdU treatment 3 of 43; control treatment 4 of 45; Fisher-Exact-Test, $n = 88$, $p = 1.0$). For further analyses we excluded these pairs, which reduced the sample size to $n = 81$. BrdU labelling had no significant effect on the number of copulations (t-Test, $t = 1.64$, $df = 79$, $p = 0.10$) or the number of double sucks (t-test, $t = -0.39$, $df = 79$, $p = 0.70$), but single sucks were observed significantly less often in the BrdU-treated group (t-test, $t = 2.77$, $df = 79$, $p = 0.007$) (fig. 7). As the function of the suck behaviour is currently unknown it is difficult to assess the implications of this result.

All 39 labelled worms had labelled sperm in the seminal vesicle, and in 13 of the 34 unlabelled worms from the BrdU treatment we observed labelled sperm in the female antrum. This proportion is lower than in Experiment 2 and is probably due to the shorter mating period (30 min instead of 2 days). Finally, in none of the control treatment worms did we ever observe labelled sperm in the female antrum. This clearly shows that the structures we identified as labelled sperm are indeed from the labelled mating partner.

DISCUSSION*Immunocytochemistry*

The original protocol published by our group, which uses a primary monoclonal mouse anti-BrdU antibody (B2531, Sigma-Aldrich, Vienna, Austria) and a secondary goat-anti-mouse FITC-conjugated antibody (F0479, DAKO, Denmark), fails to locate BrdU in sperm nuclei reliably, despite the fact that it very reliably locates BrdU in somatic and germ-line S-phase cells (Ladurner et al., 2000; Nimeth et al., 2004; Schärer et al., 2004b). Nevertheless, Ladurner et al. (2000) published a figure which clearly shows elongated sperm nuclei. They used the same protocol, which suggests that some batches of the Sigma anti-BrdU antibody can be suitable for sperm tracking purposes. However, some batches consistently failed to lead to any labelling of sperm nuclei (L. Schärer and P. Ladurner, pers. obs.). We therefore tried another primary monoclonal mouse anti-BrdU antibody (Cat. No.1170376, Roche Diagnostics, Vienna, Austria), which also failed to label sperm nuclei, even when worms were raised from the hatchling stage to maturity under continuous exposure to $50 \mu\text{M}$ BrdU (L. Schärer and P. Ladurner, data unpubl.). Such mature worms must have produced viable sperm *de novo* (as they were able to father offspring), and their sperm nuclei therefore must have contained BrdU (L. Schärer, pers. obs.).

Only after shifting to the Abcam anti-BrdU antibody (ab6326, Abcam Ltd, Cambridge, UK) were we reliably able to obtain labelled sperm nuclei. We think that a likely reason for the discrepancy between the different antibodies is that the primary Abcam antibody recognises the BrdU epitope in a way that is less sensitive to the probably highly condensed nature of the DNA in sperm nuclei. This notion

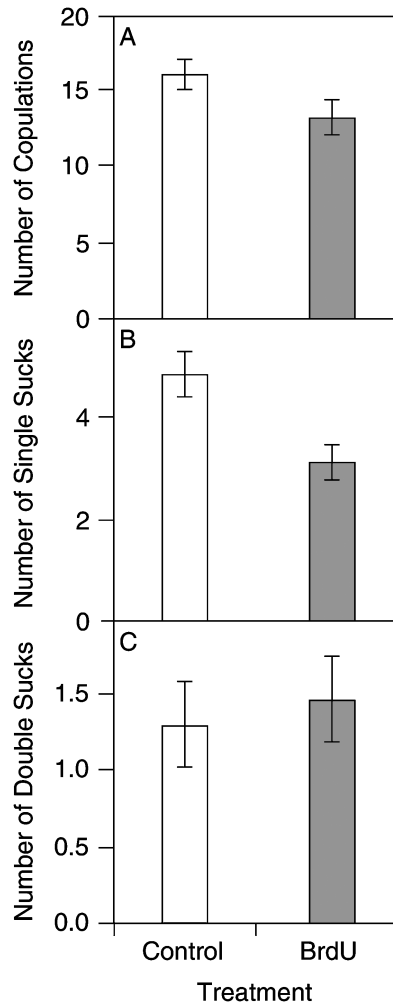


Figure 7. Effect of BrdU labelling on copulatory and postcopulatory behaviours in *Macrostomum lignano*. Control treatment (two unlabelled worms); BrdU treatment (one labelled and one unlabelled worm). See Results for statistics.

is supported by the fact that inclusion of a sperm nucleus decondensation step routinely used in spermatology to the original staining protocol (Wyrobek et al., 1990) led to some weakly BrdU-labelled sperm nuclei when we used the Roche anti-BrdU antibody.

When the right primary anti-BrdU antibody is chosen, BrdU labelling is an attractive approach to study spermatogenesis and to track the fate of the sperm of a donor in an unlabelled recipient. We think that this approach should be readily transferable to a wide range of taxa as it does not require any specific genetic knowledge. Administration of BrdU could be achieved by soaking, injection or ingestion via the drinking water. However, BrdU can be both toxic and carcinogenic

and should thus be used with care. Experiments need to be performed to establish that there are no negative effects of the label on vitality or survival, at least for the duration of the experiments.

Spermatogenesis

Our data suggest that spermatogenesis in *M. lignano* takes approximately 6 days to complete. On days 1 and 2 after labelling we mainly observed slight changes in the nuclear morphology which are probably related to the mitotic division of testicular stem cells (i.e., the spermatogonia). On day 3 after labelling we saw evidence for meiotic figures in spermatocytes, and on day 4 the products of the two meiotic divisions were evident from clusters of four spermatid cell nuclei. On day 5 the sperm nuclei started to elongate. The observed patterns on day 5 suggested that there was considerable between-individual variation in the speed of spermatogenesis. Whereas some worms had just started nuclear elongation on day 5, others seemed to have almost fully formed sperm. This observation is of interest in the context of our recent finding that sperm production rate per unit testis size can vary depending on the social situation in which a worm was raised (Schärer and Vizoso, 2006). On day 6, at least some sperm were already fully formed as mature sperm were found in the seminal vesicles. Sperm continued to be formed on days 7-9 but very few elongated sperm were observed in the testis on day 9 (data not shown).

The relatively short BrdU exposure times we used here (i.e., 0.5, 17 and 10 h for Experiments 1-3, respectively) only produced a batch of labelled sperm among numerous unlabelled sperm in the labelled donors. In order to achieve complete labelling of all sperm of an individual, a continuous exposure to low levels of BrdU would be required. As mentioned above, continuous exposure to 50 μM of BrdU is feasible for extended periods. Recent preliminary results suggest that label intensity in, and vitality and survival of, such worms are adequate.

Transfer and tracking of labelled sperm

Despite the fact that not all sperm of the labelled donors were labelled in the reported experiments, we frequently observed labelled sperm in the female antrum of worms that were mated to labelled worms. This clearly suggests that this approach is suitable to track sperm of a labelled donor in an unlabelled recipient. In cases where many sperm were transferred, it was usually not easy to determine the number of received sperm exactly. This is mainly due to the very long sperm nuclei in *M. lignano* which are about a third of the length of the total sperm, and which can form twisted strings of BrdU-positive material (high resolution LSM may allow disentangling). Alternatively, staining intensity could probably be used as a proxy for sperm number if the continuous exposure techniques outlined above could be implemented.

Mating behaviour

We found no significant effects of the BrdU label in two of three aspects of the mating behaviour, namely the copulation rate and the double suck rate. However, we observed a significant reduction in the single suck rate for the BrdU treatment group, which suggests that BrdU does at least have some effects at the concentration administered. For this method to exhibit its full potential, we need to modify the protocol in a way to ascertain that there are no (short-term) deleterious effects of BrdU on the mating performance of the worms. As already mentioned above, we think that it should be possible to reduce considerably the amount of BrdU administered while still getting a reliable immunocytochemical signal. However, we think that for a number of questions (e.g., timing of sperm storage, site of sperm storage) the neutrality of the method with respect to mating performance is less critical. Moreover, it will be mandatory in any case to assign the BrdU label in a balanced way to sperm competition offence and defence individuals in order to control statistically for any putative systematic biases on performance.

Outlook

For many questions in sperm competition research it is important to determine the proportional representation of sperm cells from one sperm donor compared to the sperm that the recipient already has in storage (i.e., to determine the S_2 -value). This requires the establishment of a method that allows the labelling of all the received sperm of the recipient. A possible approach would be to label different worms with different halogenated pyrimidines. In addition to the BrdU that we used in our study, there are two additional halogenated pyrimidines, 5-iodo-2'-deoxyuridine (IdU) and 5-chloro-2'-deoxyuridine (CldU), that are commonly used in cell cycle studies (Postberg et al., 2005). However, the specificities of the primary antibodies against these different epitopes are such that generally a maximum of two different halogenated pyrimidines can be distinguished. Nevertheless, even with only two different labels this would offer a very flexible approach to determine S_2 -values. Recent experiments in our laboratory suggest that tracking sperm labelled with CldU and IdU is possible in *M. lignano*. However, a reliable double-labelling protocol still needs to be established.

CONCLUSION

Labelling of sperm with halogenated pyrimidines represents a novel and attractive approach for tracking sperm of a donor in a recipient. We think that this approach could be readily transferred to other taxa, and that it is thus suitable for studying the mechanisms of sperm competition, sperm storage, sperm digestion and other postcopulatory mechanisms of sexual selection.

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