ethology international journal of behavioural biology

Ethology

No Plastic Responses to Experimental Manipulation of Sperm Competition *per se* in a Free-Living Flatworm

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Received: July 28, 2009
Initial acceptance: September 22, 2009
Final acceptance: November 13, 2009
(J. Kotiaho)

doi: 10.1111/j.1439-0310.2010.01746.x

Abstract

In the absence of sperm competition evolutionary theory predicts low mating rates and low ejaculate expenditure per mating, and sex allocation theory for simultaneous hermaphrodites predicts a strongly female-biased sex allocation. In the presence of sperm competition a shift towards a more male-biased sex allocation and a higher ejaculate expenditure are predicted. The free-living flatworm *Macrostomum lignano* has been shown to respond plastically in mating rate, testis size, and sperm transfer to manipulation of the social group size, a proxy of the strength of sperm competition. However, manipulation of social group size may manipulate not only sperm competition, but also other factors, such as food supply and metabolite concentration. In this study we therefore manipulated sperm competition *per se* by repeatedly exposing individuals to partners that have either mated with rivals or not, while keeping the social group size constant. Our results suggest that *M. lignano* does not have the ability to detect sperm competition *per se*, as worms experimentally exposed to the presence or absence of sperm competition did not differ in sex allocation, sperm transfer or mating behavior. A response to our manipulation would have required individual recognition, the ability to detect self-referencing tags, or tags or traces left by rivals on or in the mating partners. We first discuss the possibility that highly efficient sperm displacement may have decreased the difference between the treatment groups and then propose three alternative cues that may allow *M. lignano* to respond plastically to the social group size manipulation used in earlier studies: assessment of the mating rate, chemical cues, or tactile cues.

Introduction

Sex Allocation in Simultaneous Hermaphrodites

Sex allocation theory for outcrossing simultaneous hermaphrodites predicts that sex allocation depends on the mating group size $K + 1$, whereby K is the number of sperm donors individuals receive sperm from at the time the eggs are fertilized (Charnov 1982). When $K = 1$, there is no sperm competition and Charnov's model predicts marginal investment in sperm production and a strongly female biased sex allocation. This is a situation of maximal 'local sperm competition' (Schärer 2009) because here only related sperm are in competition with each other, in analogy to local mate competition in gonochorists (Hamilton 1967), where related males compete with each other. When $K > 1$, not only related but also unrelated sperm are competing for fertilizations, thus decreasing local sperm competition and increasing sperm competition. This leads to an increase in the optimal male allocation and thus a shift towards a more male-biased sex allocation. Consistent with this theory, studies on several

simultaneously hermaphroditic animals have reported a phenotypically plastic increase in testis size in response to increasing social group size (e.g. Raimondi & Martin 1991; Schärer & Ladurner 2003; Tan et al. 2004; Trouvé et al. 1999; reviewed in Schärer 2009), which at least in some cases is clearly associated with higher levels of sperm competition (Janicke & Schärer 2009a).

Manipulating Sex Allocation in *Macrostomum lignano*

In *M. lignano* there is a well documented effect of the social group size on testis size (e.g., Brauer et al. 2007; Janicke & Schärer 2009b; Schärer & Ladurner 2003; Schärer et al. 2004b, 2005; Schärer & Vizoso 2007; several unpublished data sets). Testis size is a meaningful measure of male allocation and sperm production (Schärer et al. 2004b; Schärer & Vizoso 2007), and bigger testes are correlated to higher sperm transfer success in *M. lignano* (Janicke & Schärer 2009a). This system thus corresponds qualitatively to the predictions of basic sex allocation theory.

Schärer & Ladurner (2003) for the first time dissected the effects of group size from density effects by simultaneously manipulating group size and enclosure size. They varied social group size by raising worms in groups of 2, 3, 4, and 8 individuals, respectively. In a fully factorial design they kept all groups in both small and large enclosures and used testis size as a measure of male allocation. They found a positive effect of group size on male allocation, but no effect of enclosure size. They interpreted the plastic increase in male allocation as a response to sperm competition, in agreement with Charnov's original prediction (Charnov 1982). As an explanation for the absence of enclosure size effects the authors speculated on a potential mechanism for individual recognition in *M. lignano*. Such a mechanism would enable the worms to differentiate between repeated encounters with the same individual and a real increase in group size. The ability to distinguish between familiar and unfamiliar mating partners may lead to a so-called Coolidge effect (first reviewed by Dewsbury 1981), which refers to an individual's decreasing propensity to mate with the same partner and a resuscitation of its sexual interest when presented with a new partner. Recently a Coolidge effect has been shown in the simultaneously hermaphroditic pond snail *Lymnaea stagnalis*. Mated snails were significantly more likely to inseminate a novel partner than their previous one (Koene & Ter Maat 2007). This behavioral response has further been documented for many other animal taxa such as beetles (Steiger et al. 2008), fishes (Kelley et al. 1999), lizards (Tokarz 1992), birds (Pizzari et al. 2003), and mammals (references in Dewsbury 1981), and it has been suggested to allow sperm reserves to be conserved for additional reproductive opportunities (Wedell et al. 2002). We here aimed to examine whether a possible differentiation between partners is the reason for the response in male allocation to social group size in *M. lignano* (see references above).

Manipulating Sperm Competition *per se*

Schärer & Ladurner (2003) expected that a higher social group size leads to a higher mating group size and therefore to a higher level of sperm competition, and this expectation was recently confirmed (Janicke & Schärer 2009a). Their observed effect of group size on testis size is consistent with the predictions regarding the optimal investment towards the production of ejaculates, summarized by Parker (1998). When males have access to more than one female in a polygamous mating system, then they should build larger testes and produce more sperm in order to counteract sperm competition as long as the fertilization chances are fair.

In the present study we compared two different situations in which individuals either encountered sperm competition every day or in which sperm competition was completely absent. We here manipulated the degree of sperm competition *per se* by using a monogamy (M) and a polygamy (P) treatment. In the M treatment we kept each flatworm with the same partner for the duration of the experiment, and transferred both worms together to a new well every day. In the P treatment we also transferred each worm to a new well, but presented it every day with a different partner out of a set of eight worms, i.e. each P replicate consisted of four pairs, newly mixed every day (see below for details). Unlike a manipulation of social group size this manipulation of sperm competition *per se* is not confounded with density and factors associated with it (e.g. food level, metabolite accumulation, encounter probability). However, it fulfils Parker's (1998) definition of sperm competition as 'competition between the sperm of two or more males for the fertilization of a given set of ova', given the fact that ejaculates of at least two, and possibly more, partners are present in the female antrum of a worm in the P treatment when it has been presented with a different partner. We hereby test whether the

response to social group size in male allocation of *M. lignano* reported by Schärer & Ladurner (2003) is based on a detection of sperm competition *per se*.

We hypothesize that (1) worms mate more often with a different partner than with a familiar partner, (2) worms transfer more sperm to a different partner than to a familiar partner, and/or (3) worms allocate more resources to testes when presented with a different partner than when presented with the same partner every day. Moreover, given that a trade-off plays between male and female allocation (Janicke & Schärer 2009b; Schärer et al. 2005) we expect a smaller ovary size and lower female fecundity as a correlated response to an increased allocation to testes in the P treatment.

Materials and Methods

Study Organism

Macrostomum lignano (Platyhelminthes, Macrostomorpha) is a simultaneously hermaphroditic free-living flatworm and a member of the meiofauna of the Northern Adriatic Sea (Ladurner et al. 2005). Experimental animals are the descendants of individuals collected near Lignano Sabbiadoro (Italy) in 2003. Mass cultures are kept in the laboratory in glass Petri dishes containing f/2 medium (Andersen et al. 2005) and with the diatom *Nitzschia curvilineata* as an *ad libitum* food source (Rieger et al. 1988). Under these conditions and at a temperature of 20°C worms reach 1.5 mm in body length and have a generation time of approx. 18 d. *Macrostomum lignano* is outcrossing (Schärer & Ladurner 2003) with frequent, reciprocal copulation, and internal fertilization (Schärer et al. 2004a). Mating rates can reach 30 times/h and microsatellite analysis has revealed multiple paternity (P. Sandner & L. Schärer, in preparation). Its transparent body wall allows to morphometrically measure the size of the paired testes and ovaries, the size of the seminal vesicle as a measure of the number of sperm ready for ejaculation and the amount of received sperm *in vivo* (Schärer & Ladurner 2003). When first mated and then isolated, individuals can store received sperm in the female antrum for up to 12 d, first laying about one egg/d and eventually running out of sperm (P. Sandner, pers. obs.). Induced variation in testis size in *M. lignano* has been shown to correlate positively with a dynamic measure of investment in sperm production (Schärer et al. 2004b), and with the number of sperm produced by a worm (Schärer & Vizoso 2007). A phenotypically plastic increase in testis size hence leads to an increase in sperm production. Higher sperm transfer can be estimated by emptier and hence smaller seminal vesicles, i.e. the sperm source, associated with higher amounts of received sperm in the female antrum, i.e. the sperm sink (Schärer & Ladurner 2003). Note that small seminal vesicles alone do not necessarily reflect low sperm production but can also be caused by recent high sperm expenditure.

Experimental Procedure

All 320 experimental animals had the same age (±1 day) because the eggs from which they hatched were laid by individuals from the stock population within 48 h. Nine days after hatching, i.e. before sexual maturation, the worms were randomly distributed from a common pool to 32 24-well tissue culture plates, such that five wells of the top line of every well contained two worms (Fig. 1). All wells were filled with 1.5 ml f/2 medium and supplied with diatoms *ad libitum*, the standard procedure in studies on plasticity of testis size in *M. lignano* (but see Schärer et al. 2005). Every four days new plates were prepared in the same way. For 2 wks all worms were transferred daily to a well located one line further down on the plate. In the M treatment each worm was transferred together with the same partner every day (see Fig. 1), and there was therefore no possibility for sperm competition. In contrast, in the P treatment each partner was transferred so that it encountered a different member of a set of eight worms every day. The transfer was done in a way that the novel partner was different from at least the ultimate and penultimate partner (Fig. 1). A recent study showed that 90% of the pairs assembled from

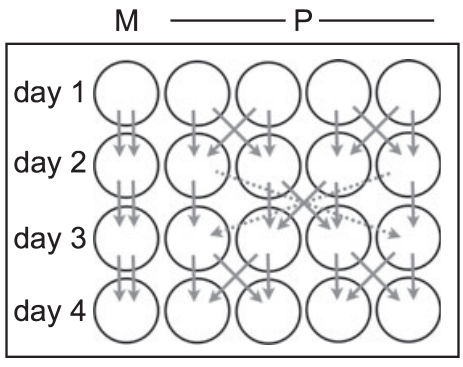

Fig. 1: Schematic representation of the experimental treatment. The fate of two worms forming one monogamy treatment replicate (M), and eight worms forming one polygamy treatment replicate (P) is depicted for four consecutive days.

two mated *M. lignano* successfully mated *and* stored sperm in the antrum of the partner when they were placed in a 24-well plate for 1 d (Janicke & Schärer 2009a). This suggests that a period of one day usually allows for at least some matings with the partner (probably many given the high mating rates), and that our manipulation therefore produced sperm competition in the P treatment replicates. *Macrostomum lignano* is able to adjust testis size within ten days when the level of sperm competition has changed (Brauer et al. 2007). Assuming that the worms in the P treatment indeed perceived higher sperm competition, higher sperm allocation for the duration of our experimental procedure, which was 14 d, was therefore expected to be reflected in a phenotypically plastic increase in testis size.

Behavioral Measurements

On day 15 of the experimental procedure, worms were not transferred to a new well but instead two worms per replicate were transferred into an observation chamber and their mating behaviour was recorded as described in detail elsewhere (Schärer et al. 2004a). Briefly, two worms were placed in a drop of 4 μl of fresh medium into an observation chamber. We filmed eight observation chambers with eight pairs each. Each chamber contained as many P replicates as M replicates and the positions of both treatments were spatially balanced. Directly after the assembly we recorded the behaviour of the worms for 1 h at 1 frame/s using a SONY DFW-X700 digital FireWire c-mount camera (Minato, Tokyo, Japan) and the software BTV Pro 5.4.1. (available at http://www.bensoftware.com/). Later we used BTV Pro 6.0b1 to score the mating rates by frame-by-frame analysis, with the observer being blind with regard to the treatment of the individual pairs.

Morphometric Measurements

After the 1 h mating trial we randomly chose one worm of each observed pair in order to measure it according to a standard procedure (Schärer & Ladurner 2003). We took digital images of the whole worm at 40×, and of both testes and both ovaries as well as of the seminal vesicle at 400× using a digital FireWire c-mount camera (DFK 41BF02, The Imaging Source Europe GmbH, Bremen, Germany) mounted on a DM 2500 compound microscope (Leica Microsystems, Germany) and using the software BTV Pro 6.0b1. For image analysis we used ImageJ 1.39u (available at http://rsb.info.nih.gov/ij/). We also estimated the amount of sperm received by the partner(s) and stored in the female antrum on a scale from 0 (no sperm visible) to 3 (many sperm visible) according to Schärer & Ladurner (2003). During all measurements the experimenter was blind with regard to the treatment groups of the worms.

Statistical Analysis

The initial sample size was $n = 32$ P and $n = 32$ M replicates. One P replicate and four M replicates were lost because of developmental problems of one of the worms (two were immature when they were measured morphometrically, two had very few sperm in their seminal vesicle and one was lacking the whole tailplate). Further, two P replicates and four M replicates were lost during the preparation of the observation chambers. The received sperm of 12 M and 15 P replicates could not be scored because of an egg in the female antrum that was ready to be deposited, and the body size of one worm could not be determined because of a missing image. This yielded a final sample size of $n = 26$ for the received sperm score (12 M; 14 P), $n = 52$ for the body size (24 M; 28 P), and $n = 53$ (24 M; 29 P) for all other variables.

Non-parametric Wilcoxon matched-pairs signed-ranks tests were used for the mating rate and received sperm score. All other variables met the assumptions of parametric tests and therefore two-sample *t*-tests could be used. Data were analyzed with JMP 7.0.1 (SAS Institute 2007).

Results

The mating rate was not significantly different between the treatment groups ($Z_{52} = 1.15$, $p = 0.25$; Fig. 2a). We did also not find a significant treatment effect on seminal vesicle size as a measure of available own sperm ($t_{52} = 1.10$, $p = 0.27$; Fig. 2b), or on the amount of received sperm as an estimate of sperm transfer ($Z_{25} = 0.33$, $p = 0.74$; Fig. 2c). Moreover, the treatment groups also did not differ significantly in body size ($t_{51} = 0.99$, $p = 0.34$; Fig. 2d), testis size ($t_{52} = 0.56$, $p = 0.58$; Fig. 2e), or ovary size ($t_{52} = 0.10$, $p = 0.92$; Fig. 2f).

Discussion

In this experiment we manipulated sperm competition *per se* in the free-living flatworm *M. lignano* and found differences in neither mating rate, received sperm score and seminal vesicles size, nor testis or

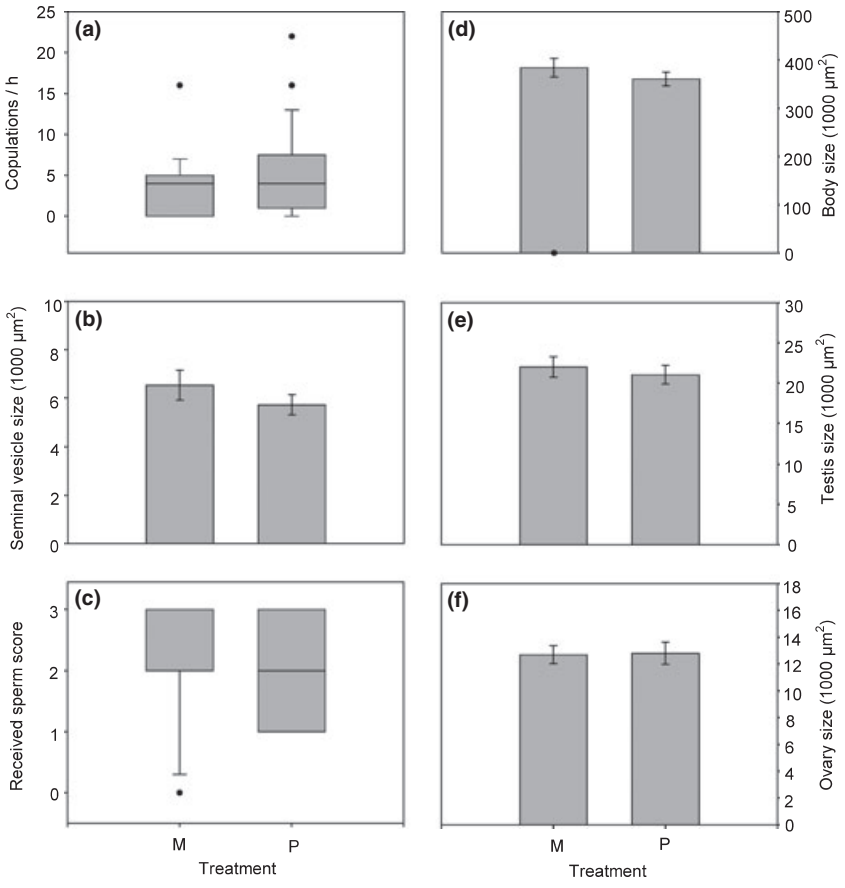

Fig. 2: Box and bar plots depicting the responses to the experimental treatment in mating rate (a), the size of the seminal vesicle as a measure of available own spearm (b), the received sperm score (c), the body size (d), the total size of both testes (e), and the total size of both ovaries (f). M refers to replicates presented with the same partner every day; P refers to replicates presented with changing patterns.

ovary size. This is in contrast to the predictions of evolutionary models and to the well-documented potential of *M. lignano* to respond to different social group sizes. In the following we first discuss the possibility of relaxed sperm competition in our P treatment. We then discuss the mechanisms for an assessment of sperm competition *per se* and finally we discuss three alternative cues for a response to sperm competition in *M. lignano*. This is done by comparisons between the experimental procedure used in this study and the manipulation of social group size used by Schärer & Ladurner (2003).

Did Sperm Displacement Relax Sperm Competition?

One possibility that might explain the lack of responses in our experiment is that sperm displacement could be highly efficient (Charnov 1996). In that case there would be little rival sperm left after just a few matings when presented with a different partner, and the matings that would follow later in the daily period would therefore entail weak sperm competition. Unfortunately, we still know little about ejaculate stratification and sperm displacement in *M. lignano*. A first study in which worms were each mated sequentially for 1 h to two partners in drops of 4 μl suggests that there is a relatively weak second male precedence in sperm transfer success (T. Janicke & L. Schärer, unpubl. data). Concerning the mating rates observed in this study the results are unlikely to be influenced by the proportion of sperm displaced. Since mating rate was measured during the first hour after the encounter with the same or different partner, sperm competition was almost certainly inevitable in the P treatment. Even when mating rates are comparable it is possible that worms in the P treatment would transfer more sperm in order to displace rival sperm, which would be seen in a higher sperm allocation. However, our observation of similar seminal vesicle sizes and received sperm scores in both treatment groups also does not indicate higher sperm allocation in the P than in the M treatment.

Possible Mechanisms for an Assessment of Sperm Competition *per se*

An adequate response to the level of sperm competition would be a Coolidge effect, i.e. an increased

propensity to mate with a novel partner and decreasing propensity to mate with a familiar partner. This can, firstly, be based on individual recognition as in burying beetles (Steiger et al. 2008) or, secondly, on self-referencing tags left on the partner's body surface during mating, as has been reported for female decorated crickets (Ivy et al. 2005). A third possible way to assess sperm competition *per se* is the detection of mating traces or tags left by rivals on the partner or in its genital tract. For instance, the nudibranch *Aeolidiella glauca* discriminates against individuals as mating partners that carry an external spermatophore stemming from a recent mating (Haase & Karlsson 2004). Moreover, there is now growing empirical evidence for correct assessment of the partner's mating state in other organisms (Anthes et al. 2006; Loose & Koene 2008; Thomas & Simmons 2009; Velando et al. 2008; Wedell & Cook 1999).

The similar mating rates we observed in both P and M treatments give no indication for a response promoted by one of these three mechanisms in *M. lignano*. As stated by Dewsbury (1981, p. 473) it is also possible that individuals, when presented with novel partners, do not mate more often but transfer more sperm per copulation. Such strategic ejaculate allocation is known from Adélie penguins that withhold ejaculates from their social partner in order to donate more sperm in extra-pair copulations (Hunter et al. 2000). However, there is also no support for either mechanism coming from the seminal vesicle size and the received sperm scores in our study. Worms did not receive more sperm in the P treatment than in the M treatment. One could argue that one would not necessarily find such a difference when most of the received sperm was displaced or lost, but the similar seminal vesicle sizes in both treatment groups give no indication to higher sperm allocation in the P than in the M treatment.

At least three other studies found no Coolidge effect or discrimination between mating states of the partner. Male decorated crickets, unlike their female conspecifics, do not identify and discriminate against previous mates (Gershman & Sakaluk 2009). The snail *Arianta arbustorum* does not adjust sperm expenditure or mating rate to the mating state of its partner (Baur et al. 1998). A recent study on the snail *Biomphalaria glabrata* shows that this snail does not discriminate former partners against novel partners in a second mating event that took place one hour after the first (Häderer et al. 2009). Beyond the lack of sensory devices and long-term memory, the authors also consider low costs of male matings as a possible reason for indiscriminate mating. Another explanation stated by the authors is that large groups and high population densities in nature make discrimination mechanisms obsolete. A similar reason might account for the absence of a Coolidge effect in male decorated crickets (Gershman & Sakaluk 2009). Here, selection for male discrimination mechanisms might be relaxed because of the strong female preference for novel males (Ivy et al. 2005).

Alternative Cues for a Response to Sperm Competition

The ability of *M. lignano* to respond to manipulations of the social group size in earlier studies can possibly hinge on differences in mating rate, chemical cues, or tactile cues, which we will discuss in turn in the following.

The first alternative trigger is the actual mating rate of an individual. When an individual gets involved in matings very frequently this might trigger a response in male allocation. It is known from *Lymnaea stagnalis*, that the fill-state of the prostate gland is detected by the brain via the penial nerve, which controls sexual activity (De Boer et al. 1997). In *M. lignano* a covariation between sex allocation and mating rate has been shown with higher mating rates in pairs formed by more male-biased individuals (Janicke & Schärer 2009b). In our study, mating rate was just like testis size not significantly different between M and P treatment groups, which is consistent with these findings. The response to sperm competition in Schärer & Ladurner (2003) study could well be mediated by higher mating rates in larger groups. However, if mating rate was correlated to encounter rate and encounter rate was higher in smaller enclosures, then Schärer & Ladurner (2003) should have detected this as an effect of enclosure size on male allocation. However, such an effect was not found.

Chemical cues can either be soluble signals or metabolites accumulating in the medium, as indicated by a study on the polychaete *Ophryotrocha diadema* (Schleicherova et al. 2006). Such conditioning of the medium – or the substrate – was minimized in this setup by the daily transfer of the worms to new wells. However, the lack of an enclosure size effect observed by Schärer & Ladurner (2003) also questions a role for soluble signals or metabolites.

Finally, tactile cues can be used by animals to sense a risk of sperm competition, e.g. when they mate with one individual and at the same time a third individual interferes with the copulating pair.

Physical contact with other individuals within a short period of time might be a similar trigger. In earlier studies sperm competition was manipulated via the social group size: no sperm competition in pairs, intermediate levels of sperm competition in groups of three or four individuals, strong sperm competition in groups of eight individuals. In those groups the rivals were allowed to compete physically with each other and the intensity of physical contact presumably increased with social group size. In the present study only the sperm of different donors were competing and there was no possibility for the worms to sense the physical presence of rivals. Tactile cues are therefore likely involved in the documented response in male allocation. However, such cues cannot be seen as strictly opposing to sperm competition as the ultimate reason for a positive response in testis size: one can control for tactile cues under laboratory conditions (this study), but in all other cases high sperm competition will coincide with high tactile cue intensity. As a consequence, tactile cues could serve as a rule-of thumb-indicator for sperm competition in the natural habitat of *M. lignano*.

Conclusions

To conclude, we did not find any behavioural or phenotypically plastic response of *M. lignano* when we manipulated the level of sperm competition *per se*. Such a response in the predicted direction is possible in our system and has been observed repeatedly and reliably when social group size was manipulated. Thus, unless our experimental treatment was ineffective due to highly efficient sperm displacement in the P treatment, we can conclude that *M. lignano* can estimate the number of partners and competitors only in their presence, e.g. mediated by tactile cues. Less likely but still possible are the perception of chemical cues or of the mating rate for an estimation of sperm competition by *M. lignano*. There is hence a need for further experiments to define the exact underlying mechanism.

Acknowledgements

We thank Jürgen Hottinger, Urs Stiefel and Viktor Mislin for technical support and Dita Vizoso for discussions. Dieter Ebert and Tim Janicke provided helpful comments on the manuscript. Nils Anthes and one anonymous referee helped to further improve the article. This project was funded by SNF (3100A0-113708) to LS.

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