



# Spermatozoa production by triploid males in the New Zealand freshwater snail *Potamopyrgus antipodarum*

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Asexual lineages derived from dioecious taxa are typically assumed to be all female. Even so, asexual females from a variety of animal taxa occasionally produce males. The existence of these males sets the stage for potential gene flow across asexual lineages as well as between sexual and asexual lineages. A recent study showed that asexual triploid female *Potamopyrgus antipodarum*, a New Zealand freshwater snail often used as a model to study sexual reproduction, occasionally produce triploid male offspring. Here, we show that these triploid male *P. antipodarum* (1) have testes that produce morphologically normal sperm, (2) make larger sperm cells that contain more nuclear DNA than the sperm produced by diploid sexual males, and (3) produce sperm that range in DNA content from haploid to diploid, and are often aneuploid. Analysis of meiotic chromosomes of triploid males showed that aberrant pairing during prophase I probably accounts for the high variation in DNA content among sperm. These results indicate that triploid male *P. antipodarum* produce sperm, but the extent to which these sperm are able to fertilize female ova remains unclear. Our results also suggest that the general assumption of sterility in triploid males should be more closely examined in other species in which such males are occasionally produced. © 2013 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, 110, 227–234.

**ADDITIONAL KEYWORDS:** gametogenesis – ploidy evolution – polyploidy – reproduction – sperm – triploid fertility.

## INTRODUCTION

The assumption that asexual lineages produce only daughters is known to be violated in a variety of taxa (Hebert *et al.*, 1989; Browne, 1992; Butlin, Schön & Martens, 1998; Smith, Kamiya & Horne, 2006; Lunt, 2008). Male production by asexual females (or maintenance of male function in hermaphrodites) could be of evolutionary importance for at least four reasons: (1) it could reduce the two-fold cost of males expected to be experienced by sexuals competing with coexisting asexuals (Neiman *et al.*, 2012), (2) it could reduce the fitness of male-producing asexual lineages compared with competing asexual lineages that produce only females (Innes, Fox & Winsor, 2000), (3) it raises the potential for gene flow among asexual lineages

and between sexual and asexual lineages (Harlan & deWet, 1975; Husband, 2004), and (4) it may assist in the maintenance of coexistence between asexual and sexual lineages (Mogie, 2011).

Asexual animal lineages are very often polyploid (Suomalainen, Saura & Lokki, 1987; Otto & Whitton, 2000), and it is generally assumed that abnormalities in testicular tissue, spermatogenesis, genetic content, and/or sperm morphology in males produced by asexual polyploid females will render these males sterile (Kawamura *et al.*, 1999; Carrell *et al.*, 2004; Mable, 2004; Hamaguchi & Sakaizumi, 2005; Rives *et al.*, 2005). One example of probable sterility in triploid males is provided by the loach *Misgurnus anguillicaudatus*; the majority of sperm produced by these males lack flagella (Oshima *et al.*, 2005). Nevertheless, fertile triploid males do occur in a variety of invertebrate and vertebrate animal taxa such as the planarian *Schmidtea polychroa*, the tench *Tinca tinca*

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(L.), and the Iberian minnow *Squalius alburnoides* (Linhart *et al.*, 2006; Sousa-Santos, Collares Pereira & Almada, 2007; D'Souza *et al.*, 2008; Hulak *et al.*, 2010).

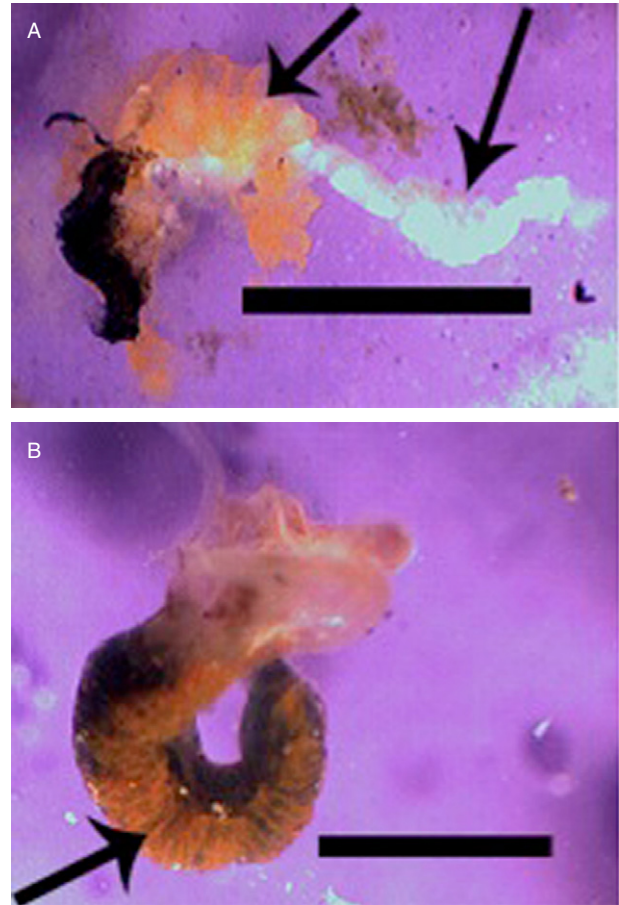
*Potamopyrgus antipodarum*, a New Zealand freshwater snail, is characterized by frequent coexistence between diploid sexual individuals (male and female) and polyploid asexual individuals (usually females) (Lively, 1987; Dybdahl & Lively, 1995). Asexual assemblages of *P. antipodarum* are genetically diverse (Fox *et al.*, 1996; Jokela *et al.*, 2003) and feature extensive within- and across-population ploidy-level variation (Neiman *et al.*, 2011). A recent study documented the existence of polyploid *P. antipodarum* with an external male genital structure at low (~5%) frequency within lake populations (Neiman *et al.*, 2011; see also Lively & Jokela, 2002) and a follow-up experiment demonstrated that these males are produced by asexual polyploid females (Neiman *et al.*, 2012).

We used triploid *P. antipodarum* that bear external male genital structures to take critical steps towards evaluating the conventional assumption that triploid males produced by asexual triploid females are sterile. There are at least five necessary components that must be in place if triploid males are to make a genetic contribution to future generations. First, triploid males must contain reproductive tissues that are able to produce sperm. Second, copulation must result in transfer of sperm to the female reproductive tract. Third, sperm must fertilize ova. Fourth, the embryos must develop into viable offspring. Fifth, the offspring must successfully mature and reproduce. We focused on step one: determining whether triploid males have testes, and if they do, whether they produce sperm. We also compared the size, morphology, and DNA content of sperm produced by triploid males with sperm produced by sexual diploid male *P. antipodarum* and used cytological techniques to examine chromosomal pairing during spermatogenesis in diploid and triploid males.

## MATERIAL AND METHODS

### IDENTIFICATION OF TESTES AND SEMINAL VESICLE TISSUE

An external genital structure, located just posterior to the head on the right side, was used to distinguish male from female *P. antipodarum* (e.g. Nelson & Neiman, 2011). Diploid male *P. antipodarum* are known to be sexual (Phillips & Lambert, 1989; Wallace, 1992), and we thus used males from an established sexual, diploid laboratory stock to locate and identify testes tissue and sperm. Snails maintained in the laboratory are kept at a constant



**Figure 1.** Both triploid and diploid males contain testes tissue that lies along the interior portion of the gut tissue and is lobular in appearance. Differences in the compaction between the tissues depicted here were an artefact of the dissection process. The testes and seminal vesicle dissected from a diploid male are depicted in A; arrows indicate testes tissue (on left) and seminal vesicle (on right); testes dissected from a triploid male is depicted in B; arrow indicates testes tissue, seminal vesicle is not visible. Scale bar = 1 mm.

16–17 °C in square containers that hold 2 L of water with a 12-h light cycle and are fed a solution of dried *Spirulina* algae three times per week. The exact location of the testes and seminal vesicle were identified via dissections, and tissue verification was conducted by use of light microscopy (Fig. 1). Dissections were conducted in saline solution so that, if present, sperm were kept alive for observations.

We compared these diploid male *P. antipodarum* with the 10 putatively triploid males found within a sample of ~1000 snails collected from deep regions (~6 m in depth) of Lake Alexandrina, New Zealand, during January 2009. We used males from this sample because previous studies indicated that males

collected from deep-water habitats in Lake Alexandrina are almost always triploid (Lively, 1987; Dybdahl & Lively, 1995; Lively & Jokela, 2002; Neiman *et al.*, 2011). These field-collected *P. antipodarum* were transported to Indiana University and maintained under standard laboratory conditions for *P. antipodarum* (described above) for approximately 6 months and then dissected to compare the internal morphology with diploid males (Fig. 1). We also used flow cytometry (methods given below) to confirm the ploidy level of these putatively triploid males.

#### SPERM COLLECTION AND MEASUREMENT

We used sperm extracted from 35 diploid and six triploid males (obtained from the same sources as above) to determine whether there were morphological and/or size differences between sperm produced by males of the different ploidy levels. We began by dissecting out the seminal vesicle and placing it in freshwater (which killed the sperm on contact), so that pictures and measurements of non-motile sperm could be taken. Sperm were extracted from the seminal vesicle with a thin needle, placed on a new glass slide, and dispersed by sliding a coverslip over the sperm-containing droplet of water. Five sperm from each individual were located using brightfield light microscopy on a Nikon E800 microscope, and an image of each sperm was taken and measured using the Metamorph imaging system (Molecular Devices, Sunnyvale, CA, USA). The head and tail of each sperm was measured length-wise from tip to tip.

#### FLOW CYTOMETRY ANALYSIS OF SPERM

We used flow cytometric analysis of sperm DNA content to determine the ploidy level of sperm produced by triploid males. Because only a small portion of sperm was used for the sperm-size measurements detailed above, we were able to flash-freeze and store the rest of the sperm and the seminal vesicle from the males used for the sperm size study for flow cytometry analysis of nuclear DNA content. We prepared samples for flow cytometry by thawing them, adding 750  $\mu\text{L}$  of cold DMSO, and then briefly grinding with a motorized grinder and pestle. We then used a mix of propidium iodide, spermine, and detergent stock solution to stain nuclear DNA (Osnas & Lively, 2006). Stained samples were filtered through a Partec Celltrix 50- $\mu\text{m}$  mesh filter and analysed with a FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA) flow cytometer at Indiana University. Somatic tissue from known diploid and triploid males was used to determine the level of refraction for diploid and triploid ranges. Sperm from diploid males were also used to determine normal refraction in the haploid range

(‘haploid standard’). We then used flow cytometry to compare the nuclear DNA content of sperm produced by triploid and diploid males and to compare those results with triploid and diploid *P. antipodarum* somatic tissue.

A random subset of samples was shipped to Amnis Corporation (Seattle, WA, USA) for analysis on an ImageStreamX Cytometer, which takes an image of each cell being examined. This method allowed us to verify that the cells being analysed were from a population of cells that were sperm. Diploid and triploid males were dissected as described above. Seminal vesicles were frozen in liquid nitrogen and shipped overnight on dry ice to Amnis. Amnis conducted cell preparation, which included DNA staining using DAPI.

Because samples were run on two different flow cytometers (at Amnis and Indiana University), we divided the mean fluorescence of each sample by the mean fluorescence of the haploid standard run on the same cytometer on the same day to calculate a standardized fluorescence value for each sample. We used these standardized values for all subsequent statistical analyses.

#### CYTOLOGICAL ANALYSIS OF TESTES TISSUE

Diploid sexual males were obtained from the laboratory lineage described above. Triploid males were born and raised in the lab, but were descended from triploid females collected from deep regions of Lake Alexandrina (–6 m in depth) in New Zealand in 2009. Testes and seminal vesicle tissues were dissected, and head tissue was used to confirm triploid chromosomal composition via flow cytometry (methods as above). We then used electron microscopy to compare the spermatogenesis process of seven diploid and seven triploid males. First, we immediately submerged the testes tissue in 100  $\mu\text{L}$  of 0.9% sodium citrate in a 1.5-mL microcentrifuge tube and gently shook the tubes for 30 min. Next, we homogenized the tissue with a plastic pestle attached to a handheld drill. After 5 min of drilling, we spun the homogenate for 10 min at 2000 r.p.m. with a desktop microcentrifuge. We carefully removed the supernatant with a pipette and then resuspended the pellet with 50  $\mu\text{L}$  of 4% paraformaldehyde. Spreading and staining techniques were the same as per Pukkila and colleagues (Pukkila & Lu, 1985; Pukkila, Skrzynia & Lu, 1992), except that spreading dishes and slides were coated with 0.9% plastic solution. Spreading dishes were allowed to dry for at least 24 h before use, but slides were used within 2–4 h after coating, which facilitated subsequent plastic removal. We also departed from Pukkila *et al.* (1992) for the wash step in dipping slides in a Kodak Photo-Flo 200 solution only 3–5



times to minimize the amount of spread lost and in omitting the water-washing step. Images from prepared slides were taken using a JEOL-1010 electron microscope. We then used these images to analyse synaptonemal complex (SC) formation in cells undergoing gametogenesis. Examining the SC and association of homologous chromosome pairs also allowed for verification of ploidy of the individual.

#### STATISTICAL ANALYSIS

The head and tail for each of five sperm from each individual were measured and recorded. Differences in sperm length (head and tail) between ploidy groups were analysed by generating a mean sperm head and tail length for each individual and then using an independent-samples *t*-test to compare the means of diploid and triploid males.

Mean nuclear DNA content of ploidy groups was obtained by calculating mean fluorescence for each individual through use of flow cytometry. We used an independent-samples *t*-test to compare these individual means between diploid and triploid males. All statistical analyses were performed with SPSS version 19 (IBM).

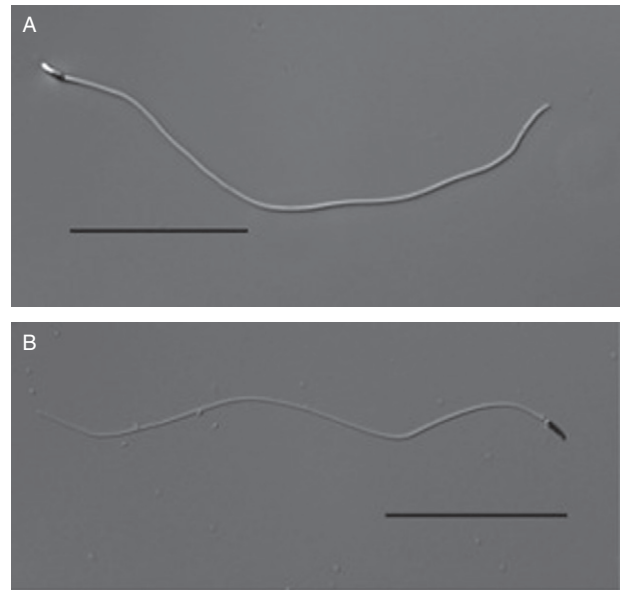
## RESULTS

#### COMPARISON OF TESTES TISSUE AND SPERM FROM DIPLOID AND TRIPLOID MALES

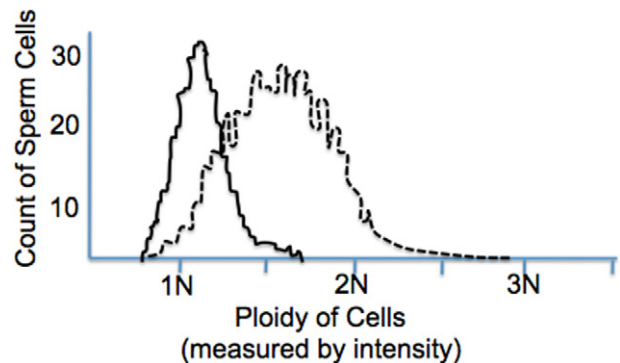
The testes of male *P. antipodarum* are internal, dark orange in colour, and reside on the dorsal surface of the soft body tissue. The seminal vesicle, a white tube structure, exits the anterior portion of the testes, which is densely packed with sperm. Triploid and diploid male testes were visually indistinguishable (Fig. 1A, B) and both exhibited mobile sperm. While the morphology of the sperm produced by diploid and triploid males did not visibly differ in structure (Fig. 2A, B), quantitative comparisons between triploid and diploid sperm revealed that triploid sperm heads and tails were ~19.2 and 14.2% larger, respectively, than their diploid counterparts. Independent *t*-tests indicated that both size differences were significant (Heads: diploid mean  $\pm$  SE =  $4.7 \pm 0.33 \mu\text{m}$ ; triploid mean =  $5.8 \pm 0.154 \mu\text{m}$ ;  $t = -10.9$ ,  $P \leq 0.001$ , d.f. = 39; Tails: diploid mean =  $95.9 \pm 0.833 \mu\text{m}$ ; triploid mean =  $111.9 \pm 1.31 \mu\text{m}$ ;  $t = -7.66$ ,  $P < 0.0001$ , d.f. = 39).

#### FLOW CYTOMETRY COMPARING DIPLOID AND TRIPLOID MALES

We used flow cytometry to analyse the nuclear DNA content of sperm produced by eight diploid males and eight triploid males. We assigned the ploidy of sperm

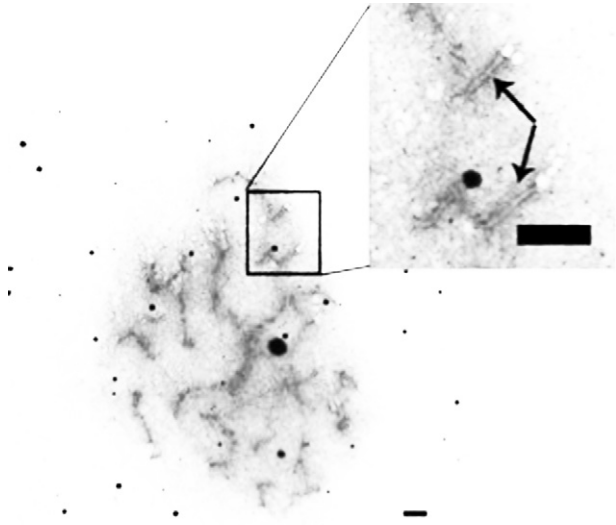


**Figure 2.** No major morphological differences exist between sperm extracted from diploid and triploid males: A, representative sperm from diploid male; B, representative sperm from triploid male. Scale bars =  $30 \mu\text{m}$ .



**Figure 3.** Dotted line indicates typical flow cytometry fluorescence from the sperm of a triploid male. Solid line indicates typical flow cytometry fluorescence from the sperm of a diploid male.

as haploid, diploid, or aneuploid; the last designation was applied to sperm with DNA content between that of known haploid and diploid standards. The analysis of sperm produced by diploid males yielded a narrow peak corresponding to haploid nuclear DNA content (Fig. 3). In contrast, analysis of sperm produced by triploid males yielded a relatively broad distribution of DNA content that ranged from haploid to diploid (Fig. 3). We also found that the nuclear DNA content of sperm from diploids ( $N = 8$ ; mean =  $0.483$ ; SE =  $0.025$ ) was significantly lower than that of sperm from triploid males ( $N = 8$ ; mean =  $0.684$ ; SE =  $0.018$ ) ( $t = -6.45$ ;  $P \leq 0.001$ , d.f. = 14).



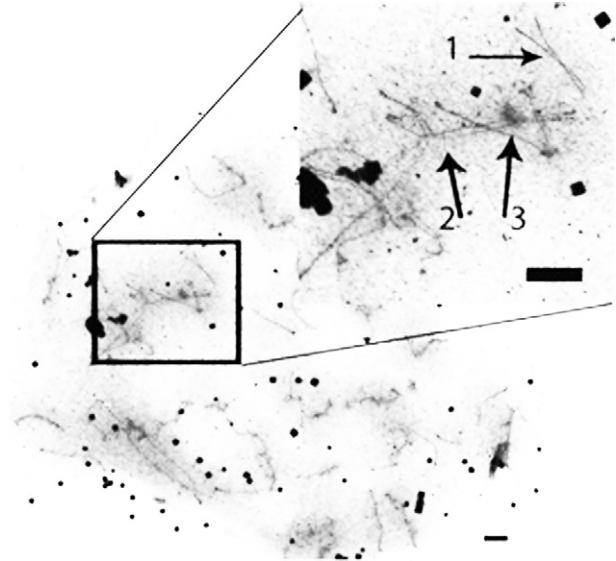
**Figure 4.** A representative spread (total examined = 15) of meiotic chromosomes from a diploid male. Arrows indicate two pairs of synapsing chromosomes. Scale bar = 2  $\mu$ m.

#### CYTOLOGICAL ANALYSIS OF TESTES TISSUE

Electron microscopic analysis of surface spreads of meiotic chromosomes showed consistent SC formation in diploid males (Fig. 4). This type of pairing is indicative of balanced chromosomal segregation that leads to haploid gametes (Zickler & Kleckner, 1999; Egozcue *et al.*, 2000; Oliver-Bonet *et al.*, 2004). Light and electron microscopy analysis through chromosomal counting confirmed that diploid individuals contain 17 pairs of chromosomes, as reported by Wallace (1992). Triploid males had mixes of bivalents and univalents as well as aberrant figures in which 'partner switching' occurs (this was observed in 11 of 12 spreads). In partner switching, an individual chromosome synapses with portions of two other chromosomes (Fig. 5, arrow 2). Abnormal synapses most likely lead to unbalanced chromosomal numbers in the resulting gametes (Jackson, 1976; Bretagnolle & Thompson, 1995).

#### DISCUSSION

We found that triploid male *P. antipodarum* produce sperm that are morphologically similar to sperm produced by diploid males, but triploids have significantly larger heads and tails. Cytometric and cytological results suggest that sperm produced by triploid males often exhibit aneuploidy, and indicate that although triploid males undergo meiosis, the chromosomal pairing is often aberrant. These latter results are of particular interest because unreduced



**Figure 5.** A representative spread (total examined = 12) of meiotic chromosomes from a triploid male. The enlarged portion shows aberrant synapsis. Arrows show bivalent pairing (arrow 1), partner switching (arrow 2), and a univalent chromosome (arrow 3). Scale bar = 2  $\mu$ m.

and/or abnormal chromosomal number in gametes is suspected to be a common source of polyploidy (Bretagnolle & Thompson, 1995; Ramsey & Schemske, 1998; Koutecký *et al.*, 2011). One relevant example comes from *Arabidopsis thaliana*, in which restitution of either the first or the second meiotic division can result in the production of offspring with higher levels of ploidy than their parents (Bretagnolle & Thompson, 1995; Köhler, Mittelsten Scheid & Erilova, 2010). A few documented cases of ploidy elevation caused by gametes with an abnormal chromosome number exist in animals. One such example comes from artificially generated triploid and natural tetraploid loaches (*Misgurnus anguillicaudatus*). Here, eggs produced by tetraploid females are fertilized with sperm (mostly aneuploid) produced by triploid males, resulting in offspring with higher levels of ploidy than their parents (Zhang & Arai, 1999). This example indicates that even in animals, aneuploid sperm should not be discounted.

Sperm competition can influence the evolution of sperm morphology, with the prediction that variation in sperm morphology will be negatively correlated with the intensity of sperm competition (Lifjeld *et al.*, 2013). While the relationship between sperm morphology and fertilization success remains unclear (Gomendio & Roldan, 1991; Briskie & Montgomerie, 1992; Birkhead & Møller, 1998; LaMunyon &

Ward, 1998; Miller & Pitnick, 2002; Snook, 2005; García-González & Simmons, 2007; Birkhead, Hosken & Pitnick, 2009), there is evidence from at least one other snail species that larger sperm may outcompete smaller sperm (Oppliger *et al.*, 2003). It is thus possible that the larger sperm produced by triploid male *P. antipodarum* may at times outcompete the smaller sperm of diploid males in the many populations in which triploid and diploid male *P. antipodarum* coexist (Neiman *et al.*, 2011).

The frequent aneuploidy of sperm produced by triploid males may mean that the fertilized eggs are less likely to develop into viable offspring than eggs fertilized by a normal haploid sperm. However, triploid males are found at about ~5% frequency in otherwise all-female and all-polyploid populations (Lively & Jokela, 2002; Neiman *et al.*, 2011). Because diploid males are absent or extremely rare in these lakes, sperm produced by triploid males represent the only possibility of fertilization success within these populations. However, because sperm morphology may be affected by environmental conditions (Gage & Cook, 1994; Hellriegel & Blanckenhorn, 2002; Amitin & Pitnick, 2006; Birkhead *et al.*, 2009), it is important to note that we cannot formally exclude the possibility that the different rearing environments of field-collected triploid males and the laboratory-reared diploid males could have differentially influenced sperm morphology. Future studies on laboratory-raised triploid males are needed to confirm that the observed differences in sperm head and tail size between diploid and triploid males hold up regardless of rearing environment.

In conclusion, we have demonstrated that triploid male *P. antipodarum* have testes and sperm, setting the stage for the possibility that these males can be a source of gene flow among asexual lineages and between sexual and asexual *P. antipodarum*. More broadly, these results show that polyploid males produced by asexual polyploid females may not be sterile. In addition, we have shown clear differences in the size of sperm produced by laboratory-reared diploid males and field-collected triploid males. We have also contributed towards understanding the cause of aneuploidy observed in the sperm of triploid males by documenting partner switching during meiotic chromosome synapsis. A complete understanding of the extent to which these males can in fact influence genetic variation and/or ploidy evolution will require the characterization of triploid male mating behaviour, the fertilization success of aneuploid sperm, especially in the context of sperm competition, and the viability and fertility of offspring that may result from fertilization with sperm produced by triploid males.

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