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Dimorphic sperm and the unlikely route to fertilisation in the yellow seahorse

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Summary

Uniquely among vertebrates, seahorses and pipefishes (Family Syngnathidae) incubate their eggs within a male brood pouch. This has contributed to a widespread, but poorly founded belief, that the eggs are fertilised using spermatozoa that are deposited directly into the brood pouch *via* an internal sperm duct. Anatomical dissections showed, however, not only that direct sperm deposition into the pouch is physically impossible, but that spermatozoa must somehow travel a significant distance (>4 mm) outside the body of the male, to reach and fertilise eggs in the pouch. Observations of courtship and mating behaviour also revealed that the pouch closes immediately after mating, and that sperm transfer must occur within a time window of no more than 6 s. In addition to this, the yellow seahorse produces extraordinarily low quantities of dimorphic spermatozoa, but is nevertheless highly fertile and can produce broods that exceed 100 embryos. The entire fertilisation process in seahorses is therefore uniquely efficient among vertebrates, yet paradoxically involves several steps that would seem to complicate, and even appear to prevent, the interaction of the gametes. Although we are still unable to describe the exact fertilisation mechanism, we speculate that spermatozoa are ejaculated into a mixture of ovarian fluid and eggs, while the male and female are in close contact. Thereafter, this mixture must enter the pouch, whereupon the spermatozoa encounter seawater. These observations also support the view, indirectly inferred in previous publications, that sperm competition in seahorses is not only non-existent but impossible.

Key words: spermatozoa, sperm transport, pouch, sperm competition, sexual selection, sperm:egg ratio.

Introduction

The unusual reproductive strategy of seahorses, whereby embryonic development occurs within a male's brood pouch, or marsupium, is well documented and justifiably famous. This has led to the widespread, but unfounded, belief that fertilisation in seahorses is 'internal' and achieved by the direct release of spermatozoa into the brood pouch cavity through an unidentified sperm duct that opens directly into the pouch interior. For example, in a review article, Kvarnemo and Simmons (Kvarnemo and Simmons, 2004) cited previous authors who suggested that once the female has transferred her eggs by inserting her ovipositor into the male's brood pouch, the male 'fertilises the eggs inside his own pouch'. In fact, anatomical evidence that spermatozoa are released through an externally directed duct was presented in a PhD thesis, written in 1967 by J. P. Boisseau (Boisseau, 1967), but to our knowledge never published as a journal article; hence, this belief has persisted to the present day. Release of spermatozoa to the external marine environment implies that fertilisation is actually 'external', in the sense that spermatozoa and eggs meet within a seawater-based milieu. At the time of mating the pouch is open to the external environment in order to receive eggs from the female; the pouch must, therefore, be filled with seawater at this time.

Although these interpretations seem logical, it is nevertheless possible that they are incorrect. There are a number of unanswered questions about sperm function and fertilisation in seahorses. For example, when exactly does the sperm–egg interaction occur? Is it possible that spermatozoa are somehow mixed with eggs in ovarian fluid rather than seawater, or are spermatozoa and eggs deposited separately into the pouch? The morphology and motility characteristics of seahorse spermatozoa have not previously been reported, and here we hypothesise that such information might provide clues to the answers. Neither is there any definite evidence about sperm:egg ratios in seahorses, although several authors have suggested that the apparent impossibility of sperm competition in these species should mean they need few

spermatozoa for successful reproduction (Parker, 1998; Stockley et al., 1997).

The aim of this study was to examine the process of mating and fertilisation in the yellow seahorse in an effort to investigate some of these unanswered questions. Initially we examined the characteristics of yellow seahorse spermatozoa and their activation responses when exposed to seawater, pouch fluid or an isotonic physiological solution. We hypothesised that the examination of sperm structure, distinguishing between 'aquasperm' [externally fertilising spermatozoa (Jamieson and Leung, 1991)] and 'introsperm' [internally fertilising spermatozoa (Jamieson and Leung, 1991)], might provide an insight into the mode of fertilisation in this species. Fish spermatozoa are immotile within the testes and are often activated by a change in their osmotic environment (Billard and Cosson, 1992; Cosson, 2004). We believed that the sperm activation responses would provide an insight into the normal environmental conditions likely to support fertilisation in the yellow seahorse. We also estimated sperm concentrations in the testes at around the time of fertilisation to validate the prediction that few spermatozoa are actually required and we re-examined the anatomy of the sperm duct and its relationship to the pouch.

Our observations supported the prediction that few spermatozoa are required for the successful fertilisation of relatively large broods, and also confirmed Boisseau's anatomical observation (Boisseau, 1967) about the location of the sperm duct. However, our observations also raise considerable uncertainty about the way in which such a small number of spermatozoa could possibly be transported from the sperm duct to the pouch without being diluted and lost. Surprisingly, the small number of spermatozoa produced by the testes showed evidence of structural dimorphism, apparently reducing still further the number of spermatozoa available to fertilise eggs.

Materials and methods

Fish

The model species used for the present study was the Indo-Pacific seahorse *Hippocampus kuda* (Bleeker, 1852), often known as the yellow or spotted seahorse. The animals were obtained from a captive-bred aquarium population that was established several years ago at Chester Zoo. The exact origins and taxonomy of this population are difficult to ascertain although it is generally regarded as *H. kuda*. Nevertheless, the *2002 Manual of Seahorse Husbandry in Public Aquaria* (Bull and Mitchell, 2002) suggests that the name *H. kuda* has often been used to describe any Indo-Pacific seahorse that could not readily be identified.

Culture and breeding of seahorses

Ten yellow seahorses (five couples), *H. kuda*, were housed in 560 l seawater aquaria; the tanks were separated into compartments (80 cm×40 cm×50 cm; length×width×height) by fine mesh dividers and one couple was maintained in each compartment. An additional 12 males used in the study were housed in the main display tank of the London Zoo aquarium where the population exhibits normal reproductive behaviour and breeding. Water was maintained at a constant temperature (26°C) and tanks were maintained under a 12 h:12 h L:D photoperiod. This photoperiod regime was adopted because this population of yellow seahorses has been shown to breed throughout the year; this indicated that photoperiod is not an important determinant of breeding activity. Furthermore, the 'Seahorse Manual' (Bull and Mitchell, 2002) recommends this photoperiod for six other seahorse species. Adult seahorses were fed four times per day with live and frozen food: *Artemia* and *Mysis* as recommended by Bull and Mitchell (Bull and Mitchell, 2002). Although embryonic seahorses possess a yolk sac while in the pouch, this normally disappears within 1 day of birth and juveniles are dependent on external nutritional sources. Juveniles were therefore fed four times per day with *Artemia* nauplii enriched with algae (*Nannachloropsis* and *Spirulina*). Normal feeding behaviour was observed in all groups of offspring from 1 day after birth.

The seahorses used for this study were part of a breeding experiment in which exact numbers of offspring were counted and inter-birth intervals noted. Correlative data about the breeding success of this particular group of seahorses was therefore available for comparison with the sperm data described here. The seahorses in our study produced broods ranging in size from 15–131 (median=60; N=12 broods).

Sperm sampling

Seahorse couples (N=5) were observed intensively and for prolonged periods to establish, and also to predict, patterns of reproduction. Mating behaviour was recorded using a video camcorder (Hi8, Sony); still images of specific behaviours were extracted from these video sequences. Courtship could be used as a guide to the optimal times for sperm collection as the testes would contain maximum numbers of spermatozoa in preparation for post-birth mating.

All sampling for the study was approved by the Zoological Society of London's Ethics Committee. Males were removed just prior to or during, egg deposition by females into the male's brood pouch, or immediately after the birth of offspring, as the testes should contain maximum numbers of spermatozoa at these times. They were sacrificed by exposure to the anaesthetic MS-222 (tricaine methanesulphonate) for approximately 10 min.

In addition to the examination of testes from the group of seahorses that had been carefully observed for mating behaviour, testes were also obtained from a group of 12 males of the same species, but housed in the London Zoo aquarium. These were sacrificed for management purposes and their reproductive status was unknown.

The seahorses were weighed, their heights measured, and the testes removed and kept on ice. Testes were divided into equal segments and macerated with known quantities (10–50 μl) of either seawater, an isotonic physiological solution (Tyrode's) (Holt and Harrison, 2002) or pouch water (fluid taken from the

pouch prior to testes dissection). The effects of ovarian fluid (10–30 μl) on sperm motility were also investigated. Ovarian fluid was obtained from two females that were sacrificed at the time they showed spawning behaviour. Whole ovaries were removed and squeezed to express ovarian fluid and undamaged eggs. Only two replications (*N*=2 males and 2 females) of this treatment were performed to minimise the number of female seahorses used. Samples (1 μl) of treated sperm suspensions were then pipetted into wells of a 12-well multitest slide (MP Biomedicals, Solon, Ohio, USA) and covered with bovine serum albumin-coated coverslips. Samples were viewed using a ×20 negative-phase contrast objective (Olympus UK Ltd, London, UK) and a green filter on an Olympus BH-2 microscope. Images were captured using a black and white video CCD camera (SPT-M108CE, Sony UK Ltd, Thatcham, UK) and recorded using a videodisc recorder (VDR-3000, Datavideo, Glossop, UK).

Sperm measurements

Video sequences were viewed using Moonlight-Elecard MPEG player (version 2.3, Moonlight Cordless, Ramat-Gan, Israel) and sperm images were traced on acetate films. Acetates were then scanned and sperm flagellar lengths, and sperm head lengths and widths were measured using Image-Pro (version 4, Media Cybernetics, Silver Spring, Maryland, USA).

Sperm duct and brood pouch opening measurement

Measurement of the distance between the sperm duct and brood pouch opening was performed with electronic callipers (RS Components Ltd, Northants, UK).

Statistical analyses

Sperm head width and length were measured and classified using hierarchical (minimum spanning tree), non-hierarchical (K-means) clustering techniques and principal components analysis (Statistica V6.1, Statsoft UK, Herts, UK). Sperm flagellar lengths were compared using a Mann–Whitney test (two-tailed).

Results

The two combined testes of each male (*N*=4 males; data for each male estimated from four replicate subsamples) contained an average total number (mean ± s.e.m.) of 304±77 mature spermatozoa. These findings were similar to preliminary observations in which a total of 160 spermatozoa (two replicates) were obtained from the testes of a big-bellied seahorse (*H. abdominalis*; *N*=1) (K. J. W. Van Look, unpublished observations). No spermatozoa at all were found upon examination of testes from the 12 males killed at random times while they showed no mating behaviour.

Examination of the morphology of yellow seahorse spermatozoa (*N*=44) revealed that they were dimorphic (designated here as Types 1 and 2), differing in both head size and flagellar length. Type 1 spermatozoa, 66% of the total, possessed small but elongated heads measuring approximately

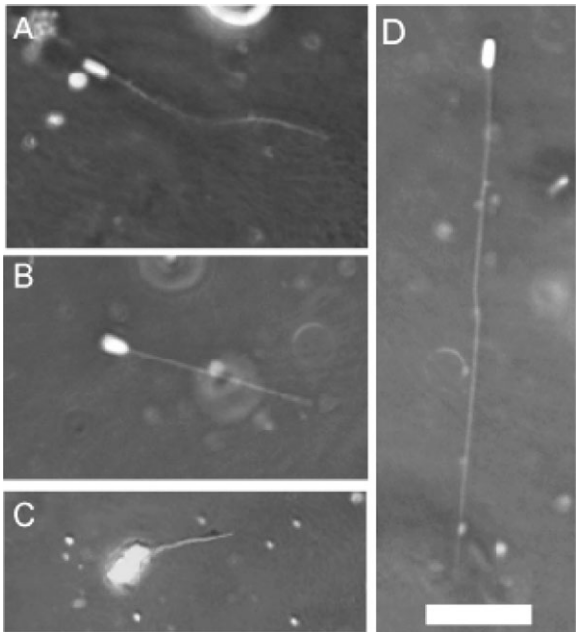

Fig. 1. Yellow seahorse spermatozoa illustrating the two types. (A,B,D) Type 1 spermatozoa of different flagellar lengths; (C) Type 2 spermatozoon with the larger head. Scale bar, 13 μm.

3.7 μm (median) in length (25% percentile=3.5 μm and 75% percentile=4.1 μm; Fig. 1A,B,D and Fig. 2B), whereas the Type 2 spermatozoa (34% of the total) possessed much larger and rounder sperm heads (median length=13.4 μm; 25% percentile=12.0 μm and 75% percentile=15.4 μm; Fig. 1C and Fig. 2B). Cluster and principal components analyses (using sperm head length, width and length:width ratio) confirmed that two groups (subtypes) of mature testicular spermatozoa were present, with one outlier which differed significantly in both sperm head length and width (*post-hoc* ANOVA analyses: length, $F_{1/42}$=135.4, P<0.001; width, $F_{1/42}$=434.2, P<0.001, N=44; Fig. 2A).

Flagellar length varied within both populations (subtypes) of spermatozoa, but also differed significantly between the two types (P=0.045, N=44). Type 1 sperm flagella varied in length between 6.3 and 69.3 μm (median length=49.3 μm, 25% percentile=34.6 μm and 75% percentile=62.6 μm) whereas flagellar lengths of Type 2 spermatozoa ranged from 13.5 to 80.4 μm (median length=25.1 μm, 25% percentile=21.1 μm and 75% percentile=48.3 μm; Fig. 2C).

Flagellar length was highly variable (>10–<60 μm) within the motile sperm population and the majority of these spermatozoa (82%) were categorised as Type 2. The motile spermatozoa (approximately one third of the total number of spermatozoa observed) were motile in seawater, pouch fluid and in the isotonic (300 mOs kg^{-1}) physiological (Tyrode's) solution. In one case the spermatozoon remained motile when seawater was added to the physiological solution in which motility was initiated. The motility activation results are summarised in Table 1.

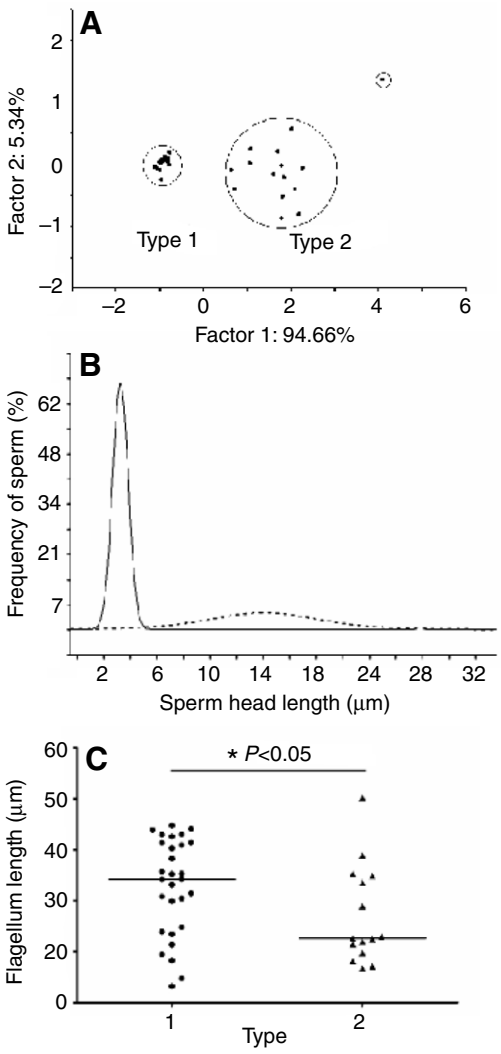

Fig. 2. Head and flagellar measurements of Types 1 and 2 spermatozoa. (A) Principal components analysis (using sperm head length, width and length:width ratio) showed that the spermatozoa fell distinctly into the two groups (Types 1 and 2) with one outlier, and both sperm head length and width were significantly different (*post-hoc* ANOVA analyses: length, $F_{1/42}$=135.4, P<0.001; width, $F_{1/42}$=434.2, P<0.001, N=44). (B) Sperm heads of Type 1 spermatozoa measured 3.7 μm (median) in length (solid line), whereas sperm heads of Type 2 spermatozoa were significantly longer (dotted line), measuring 13.4 μm (median) in length. (C) Flagella of Type 1 spermatozoa (circles) varied in length between 6.3 and 69.3 μm (49.3 μm median), whereas flagellar lengths of Type 2 spermatozoa (triangles) ranged from 13.5 to 80.4 μm (25.1 μm median). The horizontal line in each group signifies the median value; *P=0.045 (N=44) indicates a significant difference by a Mann–Whitney test (two-tailed).

The process by which the small numbers of spermatozoa reach the vicinity of the eggs in the yellow seahorse is unknown. Careful observations of mating revealed that an elaborate courtship occurred in advance of mating, but the period for completion of gamete transfer was approximately 6 s (N=5). During courtship the brood pouch of the male was fully open for around 9 s (Fig. 3), but was closed and sealed immediately upon completion of the mating process. During mating itself the male's and female's abdomens remained apposed for approximately 6 s (Fig. 3B). Anatomical observations showed that the sperm duct leading from the testes opens externally, 4.5 mm anterior to the opening of the pouch (Fig. 4). This confirmed the earlier report for the long-snouted seahorse (*H. guttulatus*) and short-snouted seahorse (*H. hippocampus*) (Boisseau, 1967).

Discussion

Seahorse spermatozoa have not previously been documented, but pipefish spermatozoa have been identified in several studies (Ah-King et al., 2006; Elofsson, 2005; Kornienko and Drozdov, 1999; Watanabe et al., 2000). However, quantitation of pipefish spermatozoa has never been attempted; for example, Watanabe et al. simply mentioned that an extremely low number of spermatozoa was found in the seaweed pipefish *Syngnathus schlegeli* (Watanabe et al., 2000).

Having found such a small number of spermatozoa in the yellow seahorse we were surprised by their apparent high degree of efficiency and tried to estimate approximate values for the sperm:egg ratio. Taking the maximum number of offspring from any single male (131 offspring born) in this study as an example would imply a minimum sperm:egg ratio of <2.5:1. Even if the number of spermatozoa (approximately 300) was underestimated by 100%, it still translates to approximately 600 spermatozoa per male and a minimum sperm:egg ratio of <5:1. These sperm:egg ratios are extremely low compared to those for other fish species. The zebrafish *Danio rerio*, which has one of the lower sperm concentrations in fish, 480,000 per ejaculate, has a sperm:egg ratio of 48,000:1, whereas that of sea trout *Salmo trutta* is 1.79×10^9:1 (Stockley et al., 1996). Sperm:egg ratios in the yellow seahorse are therefore more comparable to sperm use efficiency in Hymenoptera social insects (ants, bees and wasps) than to other fish species: in the fire ant *Solenopsis invicta* queen the ratio is 3:1 (Tschinkel and Porter, 1988) and in the honeybee queen (genus *Apis*) 3–5:1 (Baer, 2005).

Sperm competition models (Parker, 1998) and the positive relationship that exists between the risk and intensity of sperm competition, sperm numbers and gonadosomatic index across fish species (Stockley et al., 1997), predict that seahorses should have few spermatozoa. The present findings support this expectation but generate new questions about the way in which spermatozoa reach the eggs.

Our observation appears to confirm that the spermatozoa must remain viable and motile in a seawater environment, but it is unlikely that any could reach the pouch if they were reliant solely on their intrinsic motility. A possible explanation therefore may involve the spermatozoa being transported towards the brood pouch along with the eggs as they pass the sperm duct. This mechanism was proposed as an explanation for sperm transfer in the worm pipefish *Nerophis lumbriciformis*, where the female attaches her eggs to the body

Table 1. *Summary of the main characteristics and motility responses of seahorse spermatozoa*

Sperm type	% total spermatozoa	Head length*	Flagellum length*	% observed motile sperm	Media tested for sperm activation response			
					Seawater†	Physiological saline†	Ovarian fluid†	Pouch fluid†
1	66	3.7	49.3	18	+	+	+	+
2	34	13.4	25.1	82	+	+	+	+

*Lengths are expressed as median values. Percentile ranges are presented within the text.
†Qualitative motility activation response observed after spermatozoa were exposed to treatment fluids.

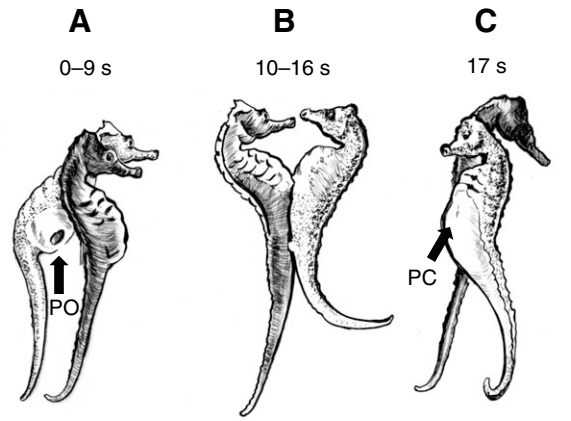

Fig. 3. Mating sequence of a yellow seahorse couple; hand drawn from video sequence. The time is shown above the images and demonstrates the duration when (A) the brood pouch remains opened (PO), (B) when gamete transfer occurs and (C) when the pouch is closed (PC).

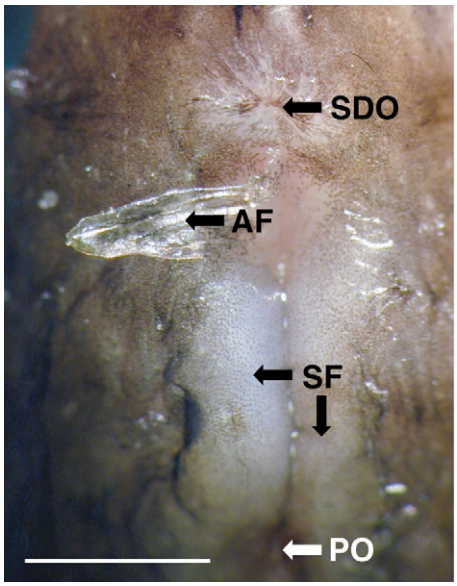

Fig. 4. Image of a male yellow seahorse showing the exteriorly directed sperm duct, which is located 4.5 mm anterior to the opening of the brood pouch. SDO, sperm duct opening; AF, anal fin; SF, skin folds; PO, pouch opening. Scale bar, 2 mm.

of the male (Monteiro et al., 2002). We also speculate that the brief (<6 s), but close contact, between male and female that accompanies egg and sperm transfer might be sufficient to create a temporary channel for sperm transport. Unfortunately, by its very nature, this speculative idea seems to defy observation and verification.

The existence of Type 1 spermatozoa in the yellow seahorse, which formed 66% of the total measured population, suggests that fertilisation in this species is internal. Internal fertilisation in the yellow seahorse would preclude the possibility of sperm competition and, moreover, would be in keeping with the minute number of spermatozoa observed. Furthermore, a low gonadosomatic index in syngnathids as a group (Kvarnemo and Simmons, 2004) also validates the lack of sperm competition in these species (Stockley et al., 1997).

The presence of more than one type of spermatozoon has previously been observed in a few fish species, seaweed pipefish (Watanabe et al., 2000) and cottoid fish *Blepsias cirrhosus* (Hayakawa and Munehara, 2004). The Type 1 spermatozoa observed here, with small and elongated heads, were similar to introsperm, and would therefore be indicative of an internal mode of fertilisation. We hypothesise that the Type 2 spermatozoa, with their much larger and rounder heads, may represent a remnant population of spermatozoa, more similar to aquasperm (Jamieson and Leung, 1991). Jamieson and Leung (Jamieson and Leung, 1991) proposed that all teleostean introsperm (which lack an acrosome) appear to be 'secondarily derived from externally fertilising aquasperm'. Viewed in this light, the Type 2 spermatozoa are likely to represent a remnant population that does not take part in fertilisation. Thus the two types of spermatozoa in the yellow seahorse may be similar to the situation in angiosperms and a variety of invertebrates, for example gastropods, spiders, centipedes and insects (Swallow and Wilkinson, 2002; Till-Bottraud et al., 2005), where the different sperm types typically correspond to the production of one fertile type and one (or more) sterile type(s). However, such dimorphism in insects is normally a feature of species in which sperm competition is a normal part of the reproductive strategy, and one of the sperm types is present merely as a 'filler' to dilate the female reproductive tract and prevent further copulations. This is incompatible with our conclusion that sperm competition does not occur in the yellow seahorse.

During mating, the brood pouch remained open for only 6 s while egg deposition occurred. During this time, seawater entered the pouch, thereby providing the hyperosmotic environment that has previously been documented in physiological studies of the marsupium (Linton and Soloff, 1964). For this reason, the environment inside the pouch cannot be regarded as truly internal because it resembles seawater. Our present observations of sperm activation and motility in seawater and pouch fluid show that physiologically 'external' fertilisation probably occurs within a physically 'internal' environment, possibly after closure of the pouch. The observation that Tyrode's medium and ovarian fluid also supported sperm activation rather complicates this scenario. It implies that seahorse sperm are rather robust in their ability to withstand extremes of osmolarity, but it also implies that the ovarian fluid that is probably released together with the eggs is also capable of motility activation.

Fertilisation in the yellow seahorse clearly presents a series of physical challenges to spermatozoa given the distance between the sperm duct and the brood pouch, and the remarkably small number of spermatozoa present. The small number of spermatozoa is, however, consistent with a lack of sperm competition in seahorses. In fact, sperm competition is anatomically prohibited in seahorses as the animals are tightly entwined for several seconds while the female deposits her eggs into the pouch. Once the eggs have been deposited, the pouch is immediately closed. There is thus no possibility for any sneaker males to be involved in the mating process. Mating with multiple males is usually regarded as bestowing direct and indirect fitness benefits, such as a higher probability of fertilisation, ejaculate nutrients and genetically more viable and resistant offspring (Møller, 1998; Wigby and Chapman, 2004). Seahorses have clearly evolved a mechanism whereby this is not feasible. In addition, the lack of sperm competition and the minimal ejaculate investment in seahorses is theoretically consistent with the high-level paternity assurance associated with their mating system (Birkhead and Møller, 1998).

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