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Fertilization Modes And The Evolution of Sperm Characteristics In Marine Fishes: Paired Comparisons of The Externally And Internally Fertilizing Species

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Abstract

Background: Fertilization modes may affect sperm characteristics, such as morphology, velocity, and motility. However, there is surprisingly little information on how fertilization mode affects sperm evolution because several factors (e.g. sperm competition and phylogeny) are intricately intertwined with this factor when phylogenetically distant species are compared. Here, we compared sperm characteristics between six externally and four internally fertilizing marine fishes from three different groups containing close relatives, taking into account the level of sperm competition. We also analysed the relationship between relative testis mass (as an index of sperm competition level) and sperm characteristics.

Results: Sperm head morphology was significantly longer in species with internal fertilization than in those with external fertilization, suggesting that a longer head is advantageous for swimming in viscous ovarian fluid or the complex ovarian structure. In addition, sperm motility differed between external fertilizers and internal fertilizers; sperm of externally fertilizing species were only motile in seawater, and sperm of internally fertilizing species were only motile in an isotonic solution. These results suggest that sperm motility has adapted according to the fertilization mode. In contrast, total sperm length and sperm velocity did not correlate with fertilization mode, perhaps because of the different levels of sperm competition. Relative testis mass is positively correlated with sperm velocity and negatively correlated with the ratio of sperm head length/flagellum length. This finding suggests that species with high levels of sperm competition have sperm that are fast and have relatively long flagella compared to head length. These results contradict a previous assumption that the evolution of internal fertilization increases total sperm length. In addition, copulatory behaviour with internal insemination may involve a large intromittent organ, but this is not essential in fish, probably due to the avoidance of water resistance.

Conclusions: We propose a new scenario of sperm evolution in which internal fertilization increases sperm head length but not total sperm length and changes sperm motility. In contrast, sperm competition affects sperm length and velocity. Our findings provide a new perspective on the evolutionary biology of sperm in fish.

Background

Spermatozoa exhibit a high degree of variation in morphology and velocity among animals [1]. Several factors have been suggested to contribute to this variation, such as fertilization modes (i.e. external or internal fertilization [2, 3]), phylogeny [4], post-mating sexual selection, including sperm competition [5], and cryptic female choice [1]. The evolution of fertilization modes and sperm competition are thought to be evolutionary forces that generate sperm diversity [2, 6, 7]. However, these factors confound each other if phylogenetically distant species are compared, and the relationship between them and sperm characteristics is obscure in many cases [1].

Generally, sperm of internally fertilizing species are believed to be longer than those of externally fertilizing species [8–10]. In addition, the relationship between the sperm components (i.e. sperm head, midpiece, and flagellum) and sperm velocity differed between species with external and internal fertilization [11]. Sperm motility of internal fertilizers in fish is similar to that of amniotic tetrapods, which are also internal fertilizers [12], but there are significant differences in motility between sperm of externally fertilizing fish and amphibians [13]. However, studies exploring the influence of fertilization modes are not as robust, possibly due to the small number of taxa that contain both externally and internally fertilizing species, and the evidence that fertilization modes affect sperm characteristics is still limited [8, 14]. In addition, the comparison of phylogenetically distant species would involve a high risk of confounding factors other than the fertilization mode [15].

Over the past 50 years, many studies on sperm competition have been documented and shown that sperm competition affects sperm morphology and speed within intra- and interspecies [1, 16]. Multiple paternity rates caused by different mating systems generate different levels of sperm competition [17–19]: low levels in monogamous species which present a dyadic relationship, and high levels in promiscuous or polyandrous species which mate with multiple partners [20, 21]. The presence of sneaker males is also a factor that increases the level of sperm competition [22, 23]. Comparative studies across taxa have shown that species with a high risk of sperm competition have longer sperm with faster swimming capabilities (e.g. fish [24], mammals [25], birds [26–28]). However, a number of studies have also shown that sperm competition level is negatively associated or not associated with sperm total length and velocity [29–32]; thus, the theory of sperm competition is a topic worthy of discussion.

In fish, while most teleost species exhibit external fertilization, a few taxa have internal fertilization with copulatory behaviour, which may have evolved multiple times [33, 34]. Fish with internal fertilization tend to possess elongated sperm heads [2, 29, 35]. Furthermore, several groups possess a unique fertilization mode called internal gametic association (IGA) with copulation [36]. The IGA process is similar to internal fertilization; however, sperm-egg fusion occurs after eggs are released into seawater. Like the internal fertilizing species, for example, some of the marine Cottidae [14, 37, 38], the sperm head of IGA species is longer than that of species with external fertilization. However, several internal fertilizing fish have oval-headed sperm [15, 39, 40], and several externally fertilizing fish have sperm with an elongated head [41–43]. Therefore, the evidence of how the fertilization mode affects sperm characteristics is still unclear, even in fish, and a comparison between close relatives is required to elucidate the effect of fertilization mode.

Furthermore, fish exhibit various mating systems and a wide range of sperm competition levels [30]. A recent meta-analysis indicated that the multiple paternity rates of internal fertilizers are higher than those of external fertilizers, suggesting that internal fertilizers can also possess high levels of sperm competition [44]. In addition, a high population density of fish increases sperm competition levels [45, 46]. High levels of sperm

competition increase sperm total length and velocity in Tanganyikan cichlid fishes [24, 47]. At the species level, sneaker males have faster sperm than territorial males in several fish (e.g. [48–51]). Furthermore, one study reported that different tactics (i.e. sneaker and courting) affect sperm midpiece morphology in internally fertilized *Xiphophorus nigrensis* [52]. Thus, bony fish are an optimal group for elucidating sperm evolution associated with different fertilization modes and sperm competition.

In this study, we investigated the effect of fertilization modes on sperm characteristics, taking into account the different mating systems and sperm competition levels in marine fish. A comparative study within the family or genus is essential for considering phylogenetic effects (e.g. [53, 54]). However, few taxa have both external and internal fertilization [15]. Thus, we compared pairs of closely related taxa from different families [47]. Three groups from a broad taxonomic range in marine fish (Group I; Pomacentridae vs Embiotocidae, Group II; Sebastinae vs Scorpaeninae, and Group III; Aulorhynchidae vs Hypoptychidae) were used in this study to assess the relationship between fertilization modes or sperm competition and sperm characteristics (Fig. 1, Additional file 1: Table S1). As data from disparate sources may be different in quality and may be erroneous [55], we caught all fish in the field (Additional file 1: Table S1) and used for the following analyses. According to the previous studies, we determined fertilization mode and estimated sperm competition levels of each species based on not only mating system, presence or absence of sneakers, the density at mating sites, and frequency of multiple mating, but also relative testes mass (RTM) we calculated (Additional file 1: Table 1). We measured sperm morphology, velocity, and motility in different solutions using six species with external fertilization and four species with internal fertilization, including species with IGA (Additional file 2: Table S2). We also examined the male intromittent organs to determine their relationship to the fertilization mode (Additional file 3: Table S3), as intromittent organs are typically projected in copulatory species (e.g. mammals, cartilaginous fish, and reptiles). We compared these characteristics within closely related species to control for phylogenetic effects, and analysed the relationships between RTM and sperm components.

Table 1 Family and species names of fish used in this study, their fertilization modes, mating systems, estimation of relative sperm competition level (ESC), and relative testes mass (RTM).

Group	Family (subfamily)	Species	Fertilization mode	Mating System	Sneaker	Density of mating site	Frequency of Multiple mating	ESC	Reference	RTM
I	Pomacentridae	*Amphiprion clarkii*	External	Monogamy	Absent	Low	Low (dyadic)	Low	[97–99]	-0.44 ± 0.05
		Chromis notata	External	MTV-polygamy[2]	Present?	High	Low (dyadic)	Medium	[100–102]	0.43 ± 0.27
		Pomacentrus nagasakiensis	External	MTV-polygamy	Present?	High	Low (dyadic)	Medium	[80]	0.33 ± 0.09
	Embiotocidae	*Ditrema temmincki temmincki*	Internal	MTV-polygamy	Present?	High	High (multiple paternity)	High	[103–105].	0.16 ± 0.26
II	Scorpaenidae (Scorpaeninae)	*Dendrochirus zebra*	External	Promiscuity	Absent	Low	Low (dyadic)	Low[5]	[106]	-0.36 ± 0.13
	Scorpaenidae (Sebastinae)	*Sebastes cheni*	Internal	MTV-polygamy[3]?	Present	High	High (multiple paternity)[3]	High	[107–110]	-0.19 ± 0.37
		Sebastiscus marmoratus	Internal	MTV-polygamy (Promiscuity)	Absent	Low	Low (dyadic)	Low	[111, 112]	-0.26 ± 0.13
III	Aulorhynchidae	*Aulorhynchus flavidus*	External	MTV-polygamy	Absent[4]	High	No data	Medium	[65, 113]	0.08
	Hypoptychidae	*Hypoptychus dybowskii*	External	MTV-polygamy	Present	High	High (multiple)	High	[64, 114]	0.19 ± 0.1
	Aulorhynchidae	*Aulichthys japonicus*	IGA[1]	MTV-polygamy	Absent	High	High (multiple)	Medium	[89, 115]	0.06 ± 0.11

[1]Internal gametic association [88]. [2]Male-territory-visiting polygamy. [3]Closely related species *S. inermis* and *S. atrovirens* showed male-territory-visiting polygamy and multiple paternity. [4]Aquarium observations found the appearance of sneaker male [116], but we referred to the field studies to arrange the condition among species, as the other studies were conducted in the field. [5]The mating system of *D. zebra* is reported to be promiscuous, but mating occurs between dyadic relationships (i.e. monogamous). Therefore, the level of sperm competition is predicted to be low.

Results

Sperm morphology

Sperm morphology was diverse among species, especially between internally (including IGA) fertilizing and externally fertilizing species (Fig. 2). In Group I, the total sperm length of internally fertilizing species was significantly longer than that of externally fertilizing species (Fig. 3A, Additional file 4: Table S4). On the other hand, there was no apparent relationship between reproductive modes and total sperm length in Groups II and III. A significant statistical difference in total sperm length was detected in Group II; however, the difference in total sperm length of *Dendrochirus zebra* and *Sebastiscus marmoratus* was slight, and the pair seemed to have the same levels of sperm competition (Fig. 3B, Additional file 5: Table S5). Furthermore, *Sebastes cheni,* which seems to have higher levels of sperm competition, had longer sperm than *D. zebra* and *S. marmoratus*. The same tendency was detected in Group III, resulting in species with a high level of sperm competition producing longer sperm than species with a medium level of sperm competition (Fig. 3C, Additional file 6: Table S6).

Sperm head morphology was different between externally and internally fertilizing/IGA species in all groups. The sperm heads of internal fertilizers/IGA species were slenderer than those of external fertilizers, which showed spherical or oval morphology (Fig. 3D, E, F, Additional file 4, 5, 6: Table S4, S5, S6). One exception was the sperm of *Hypoptychus dybowskii,* which had a slightly longer head and the same head ratio as the IGA species *Aulichthys japonicus* (Fig. 3F, Additional file 6: Table S6).

The ratio of midpiece length to width showed similar results with head morphology in groups I and III, in which internally fertilizing/IGA species had a longer midpiece than externally fertilizing species (Fig. 3G, I, Additional file 4, 6: Table S4, S6). A statistically significant difference in midpiece morphology was found in Group II (Fig. 3H, Additional file 5: Table S5), but not in Group II between externally fertilizing *D. zebra* and internally fertilizing *S. cheni*.

Sperm motility and swimming velocity

Sperm motilities in different solutions were different between external and internal fertilizers/IGA in all three groups (Table 2). Sperm of externally fertilizing species were motile in seawater but immotile in an isotonic solution that imitated ovarian fluid. Only the sperm of *Aulorhynchus flavidus* were activated in both seawater and isotonic solutions (Table 2). In contrast, sperm of internally fertilizing species, including IGA species, were motile in the isotonic solution but not in seawater. We did not obtain consistent results between sperm velocity and fertilization mode. In Group I, the sperm velocity of *Amphiprion clarkii,* in which the sperm competition level was low, was lower than that of the other species (Fig. 3J, Additional file 4: Table S4). In Group II, *S. cheni* and *S. marmoratus* had faster sperm than *D. zebra* with low levels of sperm competition (Fig. 3K, Additional file 5: Table S5). The results showed that there was no significant difference in the sperm velocity of fish in Group III, which had a similar reproductive mode and sperm competition (Fig. 3L, Additional file 6: Table S6).

Table 2 Sperm motility in seawater and isotonic solution of 10 species used in this study.

Group	Species	Fertilization mode	Sperm motility	
			Seawater	Isotonic solution
I	*Amphiprion clarkii*	External	Motile	Immotile
	Chromis notata	External	Motile	Immotile
	Pomacentrus nagasakiensis	External	Motile	Immotile
	Ditrema temmincki temmincki	Internal	Immotile	Motile
II	*Dendrochirus zebra*	External	Motile	Immotile
	Sebastiscus cheni	Internal	Immotile	Motile
	Sebastes marmoratus	Internal	Immotile	Motile
III	*Aulorhynchus flavidus*	External	Motile	Motile
	Hypoptychus dybowskii	External	Motile	Immotile
	Aulichthys japonicus	IGA[1]	Immotile	Motile

[1]Internal gametic association.

Relative testes mass (RTM) and sperm characteristics

Sperm swimming velocity was significantly and positively correlated with RTM (Fig. 4A, Table 3). We also found that the ratio of sperm head length to flagellum length was negatively correlated with RTM (Fig. 4B, Table 3). No correlation was detected in the other components of sperm, except for midpiece width (Table 3). We also found no significant relationship between total sperm length and velocity or the ratio of head/total sperm length and velocity (Table 3).

Table 3 The relationships between sperm velocity and sperm components and between sperm components and relative testes mass (RTM). N = 10 species. Bold letters show statistically significant correlations.

Response	Predictor	r	t	P
Sperm velocity	Total sperm length	0.007	0.02	0.98
	Head length / flagellum length	-0.37	-1.14	0.29
	Head length	-0.31	-0.91	0.39
	Midpiece length	0.12	0.35	0.74
Total sperm length	RTM	0.32	0.96	0.37
Flagellum length	RTM	0.38	1.15	0.29
Head length	RTM	-0.43	-1.34	0.22
Head width	RTM	-0.35	-1.06	0.32
Midpiece length	RTM	0.17	0.49	0.64
Midpiece width	**RTM**	**-0.74**	**-3.10**	**0.015**
Head length / head width	RTM	0.09	0.25	0.81
Midpiece length / midpiece width	RTM	0.32	0.96	0.36
Head length / flagellum length	**RTM**	**-0.67**	**-2.54**	**0.034**
Sperm velocity	**RTM**	**0.71**	**2.82**	**0.023**

Genital morphology

As expected, external fertilizers did not have an apparent intromittent organ (Fig. 5A, B, E, H, Table 4); however, a relatively sizeable genital papilla appeared in *Pomacentrus nagasakiensis* after pressing the abdomen (Fig. 5C). Of the internal fertilizers and species with IGA, *S. marmoratus* and *A. japonicus* each had a large and slender intromittent organ, which was retracted in the abdominal cavity unless the abdomen was pressed (Fig. 5G, I). The other internally fertilizing fishes, such as *Ditrema temmincki temmincki* and *S. cheni*, had no noticeable copulatory organ, although the small genital papilla was present (Fig. 5D, F).

Table 4 Summary of reproductive modes and relative genital length in male fish used in this study.

Group	Species	Reproductive modes	Relative genital length (n)	Statistics
I	*Amphiprion clarkii*	External	-0.42 ± 0.17^{a} (4)	F = 23.45, P < 0.0001
	Chromis notata	External	-0.26 ± 0.04^{a} (3)	
	Pomacentrus nagasakiensis	External	0.63 ± 0.14^{b} (3)	
	Ditrema temmincki temmincki	Internal	-0.2 ± 0.23^{a} (5)	
II	*Dendrochirus zebra*	External	-0.9 ± 0.28^{a} (2)	F = 36.64, P < 0.0001
	Sebastes cheni	Internal	-0.31 ± 0.11^{b} (6)	
	Sebastiscus marmoratus	Internal	-0.06 ± 0.06^{c} (6)	
III	*Aulorhynchus flavidus*	External	NA	
	Hypoptychus dybowskii	External	-0.34 (1)	
	Aulichthys japonicus	IGA	0.99 ± 0.02 (6)	

Different superscripts indicate significant differences among species in each group (ANOVA, followed by Tukey's HSD test, $P < 0.05$). Brackets show the number of individuals. NA: We lost the picture of cloaca, but there were no or few genitals.

Discussion

Previous studies in fish have suggested that internal fertilizers, including IGA species, have sperm with a more elongated head than the external fertilizers [2, 14]. Notably, our results suggest the same tendency as in previous studies, even when close relatives are compared. Thus, different fertilization patterns could be a strong evolutionary force affecting the sperm head, and we clearly showed the tendency of the effect of fertilization modes on sperm head morphology. Several studies indicate that sperm head morphology affects sperm swimming behaviour [56, 57]. For example, a narrow head reduces drag so that slender-headed sperm can travel easily through the viscous ovarian fluid [25, 58, 59]. This is possible because the viscosity of the ovarian fluid is 2–3 times higher than that of water [60]. Furthermore, the theoretical model indicates that the drag of the head ratio around two (i.e. 2:1 head length: head width ratio) is lower than the spherical morphology (i.e. 1:1 ratio), although a more slender head (over 4:1 ratio) increases the drag more than a sphere [61]. In this study, the head ratios of internal fertilizers were between 1.29 and 2.83 (see Additional file 4, 5, 6: Table S4, S5, S6). Therefore, the hypothesis that the sperm head of species with internal insemination is elongated to adapt to a viscous environment is likely to be applied to the fish used in this study. Generally, the female ovary is somewhat complicated and narrow, and sperm motility may be obstructed more in the ovary than in seawater, which is an unobstructed space. Therefore, an elongated head may be suitable for propulsion in the complicated ovarian environment.

Nevertheless, there was no statistically significant difference between the head morphology of externally fertilizing *H. dybowskii* and *A. japonicus* with IGA in Group III. This result coincides with the observation of sperm morphology in the Gasterosteoidei [43, 62]. In Baikal sculpins, the sperm of externally and internally fertilizing species also have the same head morphology, while the head volume is smaller in internally fertilizing species than externally fertilizing species, which may be an adaptation to viscous environments [15]. A smaller sperm head has a lower drag force against the solution compared to a larger head [63]. In this study, there was no difference in the head cross-sectional area between *H. dybowskii* and *A. japonicus* (Additional file 6: Table S6, LMM, $c^2 = 2.032$, $P = 0.154$). Although the reproductive behaviour of *A. flavidus* is similar to that of *H. dybowskii* [64, 65], *A. flavidus* has oval-headed sperm. The reason why sperm head morphology was similar between *H. dybowskii* and *A. japonicus* is still unknown, and further detailed research is required.

We showed that total sperm length and velocity varied independently of the reproductive modes. Comparative studies among a wide range of taxa show that internal fertilizers tend to have longer sperm than external fertilizers [8–10]. However, comparisons among phylogenetically distant species possess the risk of confounding factors other than different fertilization modes, and these factors should also influence sperm morphology and speed. Our comparisons with close relatives indicated that fertilization modes did not affect sperm length and velocity, even though their swimming environments were different. Several studies among related species have shown that the total sperm length and velocity increase as sperm competition levels increase [24, 25, 27, 47, 66]. Our pair comparison of total sperm length was partially consistent with these studies; species with a low level of sperm competition had shorter sperm than species with high levels of sperm competition (e.g. *A. clarkii* vs *D. temmincki temmincki* and *D. zebra* vs *S. cheni*), although some pairs did not match this assumption (e.g. sperm length; *A. clarkii* vs *C. notata* and *P. nagasakiensis,* velocity; *S. cheni* vs *S. marmoratus*). The long flagellum (but not extra-long flagellum) is advantageous for swimming speed [67], such that species with high levels of sperm competition may have longer flagella [68]. We also showed that relative testes mass (RTM), the proxy of sperm competition, did not correlate with total sperm length directly; however, the ratio of head to flagellum length was negatively correlated with RTM, suggesting that sperm competition promotes relatively longer flagella to head length. Sperm swimming speed was also positively correlated with RTM. Thus, relatively longer flagella and velocity are likely to match the theory of sperm competition [68, 69], although there was no relationship between sperm velocity and sperm length or the head/flagellum length ratio. Therefore, these characteristics may be influenced by sperm competition but not by the different fertilization modes.

There was no apparent relationship between midpiece morphology and the fertilization modes. The midpiece contains mitochondria, which generate energy; thus, midpiece volume is likely to be related to sperm velocity [25, 70–73], and several studies have shown that midpiece size is positively correlated with different levels of sperm competition [25, 70, 71, 74]. However, our studies showed that the midpiece size was not related to the estimation of relative sperm competition levels. Sperm velocity also did not correlate with the midpiece size. Midpiece morphology may be a confounding factor, even though closely related species were compared.

We showed that sperm motility strongly depended on fertilization mode. In this study, the sperm of externally fertilizing species was motile only in seawater, except for *A. flavidus*, which was motile in both seawater and isotonic solution. This result suggests that marine fish with external fertilization have adapted to the external environment, that is, seawater, where eggs are fertilized. However, similar to the motility of *A. flavidus,* several externally fertilizing marine/freshwater sculpins have motile sperm in both seawater/freshwater and isotonic solutions [15, 37, 75]. Thus, some species with external fertilization might have the potential to swim in ovarian fluid, generating the foothold of internal fertilization [15]. On the other hand, all sperm of internally fertilizing species, including IGA species in this study, swam only in the isotonic solution but not in seawater. Similar results were found for a marine sculpin with IGA [76, 77]. In addition, sperm of freshwater fish with viviparity were only motile in isotonic solution but immotile in low osmotic solution in Baikal sculpin [15] and swordtail [78]. Therefore, species with internal insemination may have sperm adapted to the internal environment. A previous study has shown that the sperm of marine fish have a swimming potential for a comparatively more

comprehensive range of osmolarity than freshwater fish [79]. However, little information is available on the sperm motility of marine fish subjected to internal fertilization. Based on a previous study on externally fertilizing marine fish and our results, we conclude that the sperm motility of marine fish with internal fertilization and IGA is restricted in an isotonic solution and no longer needs motility in seawater because of the evolution of fertilization modes.

In this study, the intromittent organ was not perfectly associated with fertilization modes because some internal fertilizers did not have a large intromittent organ suitable for copulation. Our study suggests that copulatory organs might not be necessary for internal insemination in fish, but externally fertilizing *P. nagasakiensis* has a small genital papilla, which it protrudes to rub over the eggs during mating [80]. Furthermore, there was a significant difference in the relative genital length between internally fertilizing *D. temmincki temmincki* and *P. nagasakiensis*. In tetrapod vertebrates, the presence of an intromittent organ is related to copulatory behaviour in mammals and reptiles but not in amphibians and birds [81]. The length of the intromittent organ is positively associated with high levels of sperm competition in waterfowl and mammals [82, 83]. *Aulichthys japonicus,* which seems to exhibit medium levels of sperm competition, has a large copulatory organ. However, the large genitalia might obstruct swimming, resulting in the fish retracting their genital papilla into their bodies. Other internally fertilizing fish used in this study may lack large intromittent organs for the same reason. This assumption may explain why amphibians and birds generally do not have an intromittent organ; it would impede swimming as well as flying.

Conclusion

In this study, we compared the sperm morphology and motility of external fertilizers and species with internal insemination among close relatives to control for phylogenetic effects. We clearly showed that internal fertilization, including IGA, influences sperm head morphology and motility in the extracellular environment. Although there were a few exceptions, total sperm length and velocity might be associated with sperm competition, but not fertilization mode. Previous studies suggested that internal fertilizers have longer sperm than external fertilizers [8–10] . However, our results propose a new theory: internal insemination with internal fertilization and IGA did not affect total sperm length. Our findings reveal, for the first time, that the evolutionary force of fertilization modes affects sperm characteristics by comparing closely related species in marine fish.

Materials And Methods

Fish sampling and measurements of genital length and testes mass

We selected six species of externally fertilizing fish, three internally fertilizing species, and one IGA species. They were assigned to three closely related species groups (Groups I, II, and III) according to their phylogenetic relationships (Table 1, Fig. 1; [84–87]). Group I consisted of externally fertilizing anemonefish (*Amphiprion clarkii*), damselfish (*Chromis notata* and *Pomacentrus nagasakiensis*), and internally fertilizing surfperch (*Ditrema temmincki temmincki*). Group II consisted of externally fertilizing lionfish (*Dendrochirus zebra*) and internally fertilizing rockfish (*Sebastes cheni* and *Sebastiscus marmoratus*). Group III consisted of externally fertilizing tube-snout (*Aulorhynchus flavidus*), sand-eel (*Hypoptychus dybowskii*), and Japanese tube-snout (*Aulichthys japonicus*) with IGA [88, 89].

All fish were collected by hand nets using SCUBA, hook and line, and gillnet during their reproductive season in the nearshore waters of Japan and off the west coast of California, USA (Table S1). The specimens were anaesthetized with MS-222 (200 mg/L), and euthanized via cervical transection, by snipping the spinal cord between cervical vertebrae 1 and 2 with sharp scissors, according to American Veterinary Medical Association. Their total length, standard length (mm), and body mass (g) were measured. We photographed the genital papilla by gently pressing the abdomen to observe the male genital morphology. The length of the genital papilla (mm) from the basal position to the tip was measured using photographs and Image J ver. 1.50 (National Institutes of Health, USA). The testes or ovaries of the specimens (g) were taken out and weighed.

Sperm motility and velocity

Extracted testes and ovaries were placed on a Petri dish on ice to prevent contamination with urine, mucus, and seawater. Semen or ovarian fluid, which contains sperm, was collected by incising the posterior region of the testes and used for subsequent analyses. We immediately diluted semen or ovarian fluid (< 1μL) in natural seawater or isotonic solution (30 μL) that imitated ovarian fluid (150 mM NaCl, 10 mM HEPES, pH 8.0, [76, 90]) on glass slides coated with 1% bovine serum albumin. Sperm motility was observed using phase-contrast microscopy (DSM-IIH-104, Daiko Science, Japan), equipped with a digital CCD camera (MTV-63WIN, Mintron Enterprise, Taipei, Taiwan) and recorded using Blackmagic Video Recorder software ver. 1.0.1 (Blackmagic Design, Australia) and AmScope ver. 4.8 (AmScope, USA).

We measured the sperm velocity of externally fertilizing species in seawater and internally fertilizing species in an isotonic solution. Water temperature was set according to the seawater temperature at which the fish were collected (Additional file 1: Table S1). Sperm trajectories were recorded for 1 s (30 frames/s) using the cell motility analysis software (BohBoh ver. 4.51 J, BohBoh soft, Japan) within 0–30 s from the videos and sperm curvilinear velocities were measured using sperm trajectories and Image J ver. 1.50i.

Sperm morphology

Semen or ovarian fluid was fixed with optimal fixative (2.5% glutaraldehyde, 0.45 M glucose, 60 mM HEPES, following [90]). The sample was placed on a microscope slide, and the images of sperm morphology were obtained using a differential interference microscope (BX50, Olympus, Japan) equipped with a digital colour CCD camera (DMK 33UX174, The Imaging Source, Germany) and IC capture software ver. 2.4 (The Imaging Source, Germany), or differential interference microscope (BX53, Olympus, Japan), equipped with a digital colour CCD camera (DP73, Olympus, Japan) and CellSens Standard software ver. 1.9 (Olympus, Japan). We measured the morphology of sperm components (total sperm length, flagellum length, head length, head width, midpiece length, midpiece width, head length/width, midpiece length/width, and head length/total sperm length) from the images using Image J ver. 1.50i. The head length/width and midpiece length/width ratio were used to determine the head and midpiece morphology.

Data analysis

Before analysis we eliminated outliers from the datasets using IBM SPSS Statistics ver. 23.0 (SPSS, Inc., Chicago, USA) to avoid spurious effects [40 out of 1079 (3.7%) sperm for morphology and 32 out of 1312 sperm (2.4%) for velocity, respectively]. We compared sperm components, including sperm total length, head length and width, midpiece length and width, and velocity among external fertilizers and internal fertilizers within the same Group (Group I, II, or III). Analyses were conducted using linear mixed models (LMMs) as implemented in the lme4 package [91] with R 3.4.1 [92]. In the models, each species was treated as a fixed effect and individual fish as a random effect because we measured sperm characteristics of multiple sperms per male. Sequential Bonferroni multiple comparison methods [93] were applied to control the family-wise error rate among the species. Differences were considered statistically significant at $P < 0.05$.

Sperm competition levels of each species were estimated based on not only mating system and population density, according to previous studies (Table 1), but also relative testis mass (RTM). RTM is generally used as a proxy for sperm competition; species with high levels of sperm competition are predicted to have larger testes than species with low levels of sperm competition [6, 31, 94–96]. We calculated the residuals (i.e. RTM) from the regression line estimated by an LMM on the relationship between log soma mass (body mass – testes mass) and log testes mass, setting each species as a random effect (Additional file 7: Fig. S1). Simple regression analyses were performed between RTM and sperm characteristics, both averaged by species, to assess whether the species in which males invested more in testes would have superior sperm (i.e. faster-swimming sperm), and Pearson's coefficients (r) were calculated.

To assess whether males of internally fertilizing species had elongated intromittent organs, we calculated the relative genital length. The relative genital length was estimated as the residuals from the regression line estimated by an LMM on the relationship between log standard length and log genital length, and each species was set as a random effect (Additional file 8: Fig. S2). Mean relative genital length was compared using one-way analysis of variance (ANOVA), followed by Tukey's HSD test for multiple comparisons.

Abbreviations

IGA, internal gametic association; RTM, relative testes mass; ESC, estimation of relative sperm competition; LMM, linear mixed model

Declarations

Ethics approval and consent to participate

The study was carried out in compliance with the ARRIVE guidelines. Our research was conducted with adherence to the Scientific Collecting Permit, State of California, Department of Fish and Wildlife (permit No. SC-13856). Research permission was also obtained from the Fisheries Cooperative Association of Sado, Japan. All field and laboratory procedures complied with the Animal Care Committee of Osaka City University.

Availability of data and materials

The datasets used and analysed during the current study are available are on Additional file 2_TableS2.xlsx and Additional file 3_TableS3.xlsx

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TI and SA designed the study, analysed the data, and wrote the first draft of the manuscript; TI, MM, HM, YK, MH, and SA conducted the sampling in the field; MM, KI, and KS assisted sperm motility and morphological analyses; All authors read and revised the manuscript, gave their final approval for publication.

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Figures

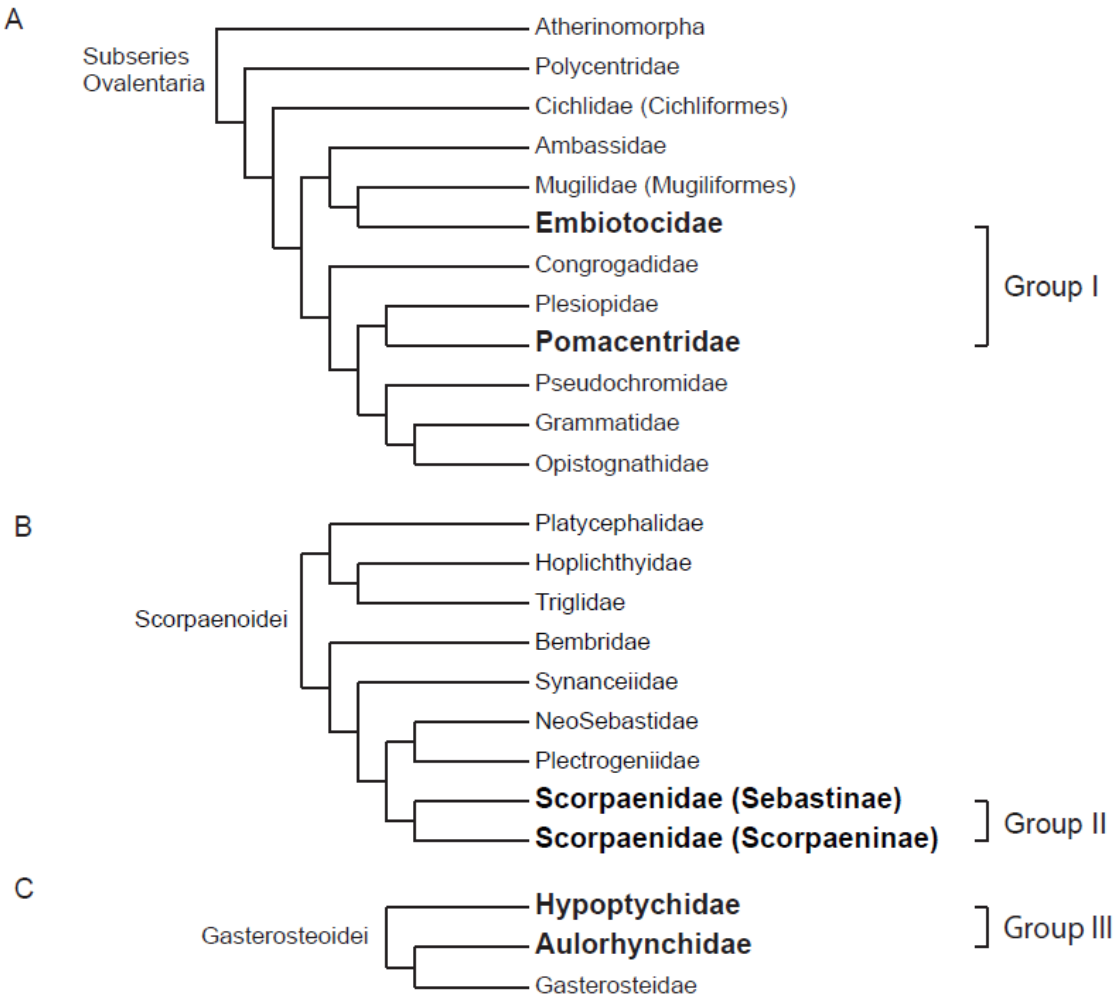

Figure 1

Interfamilial relationship of phylogeny of (A) Ovalentaria, (B) Scorpaenoidei, and (C) Gasterosteoidei. Each phylogenetic tree was based on [85], [86], and [87] , respectively. Bold letters show the families used in this study.

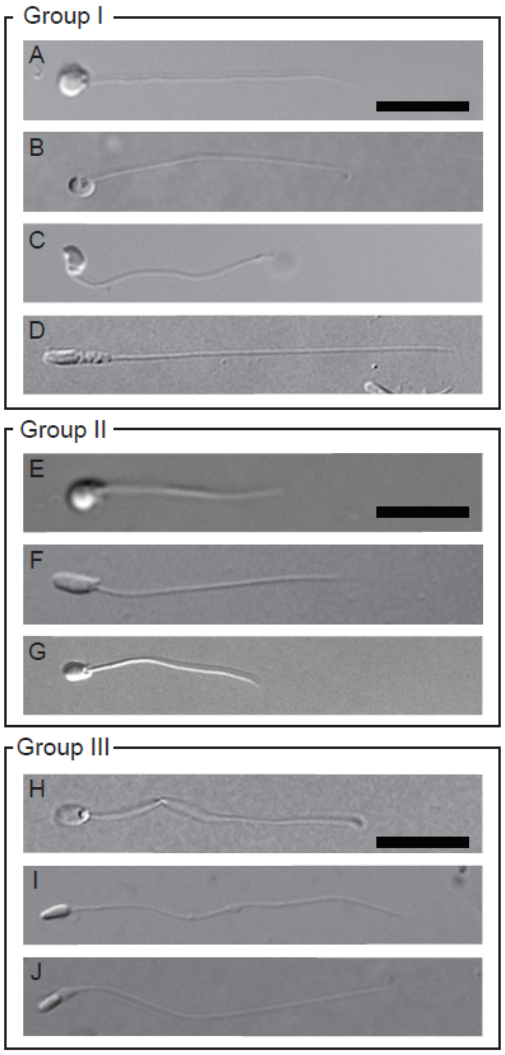

Figure 2

Sperm of species with external fertilization and species with internal insemination. (A) Amphiprion clarkii. (B) Chromis notata. (C) Pomacentrus nagasakiensis. (D) Ditrema temmincki temmincki. (E) Dendrochirus zebra. (F) Sebastes cheni. (G) Sebastiscus marmoratus. (H) Aulorhynchus flavidus. (I) Hypoptychus dybowskii. (J) Aulichthys japonicus. Scale bars show 5 mm.

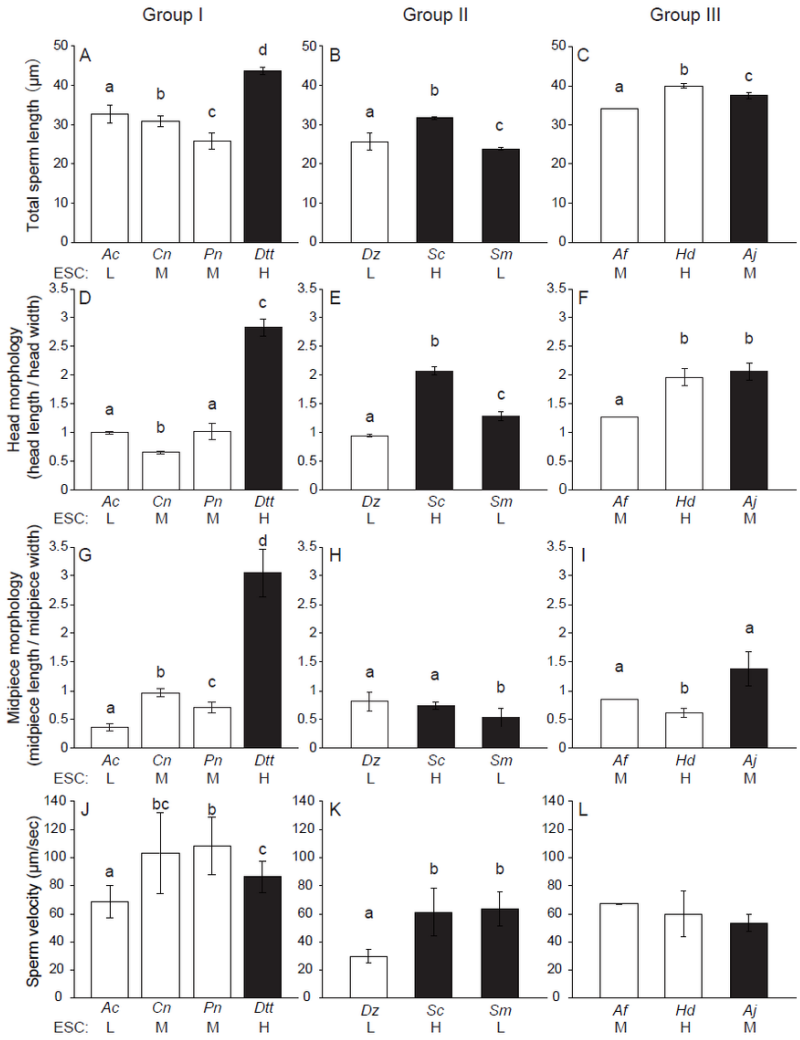

Figure 3

Differences in sperm morphological characteristics (mean ± SD) of three groups with different fertilization modes. Total sperm length in Group I (A), Group II (B), and Group III (C). Head ratio in Group I (D), Group II (E), and Group III (F). Midpiece ratio in Group I (G), Group II (H), and Group III (I). Sperm swimming velocity in Group I (J), Group II (K), and Group III (L). White bars and black bars indicate species with external fertilization and species with internal insemination, respectively. Estimated relative sperm competition levels (ESCs) are also shown under the bar: low (L), medium (M), and high (H). Ac, Amphiprion clarkii; Cn, Chromis notata; Pn, Pomacentrus nagasakiensis; Dtt, Ditrema temmincki temmincki; Dz, Dendrochirus zebra; Sc, Sebastes cheni; Sm, Sebastiscus marmoratus; Af, Aulorhynchus flavidus; Hd, Hypoptychus dybowskii; Aj, Aulichthys japonicus. Different letters indicate significant differences between species (LMMs with sequential Bonferroni correction, $P < 0.05$).

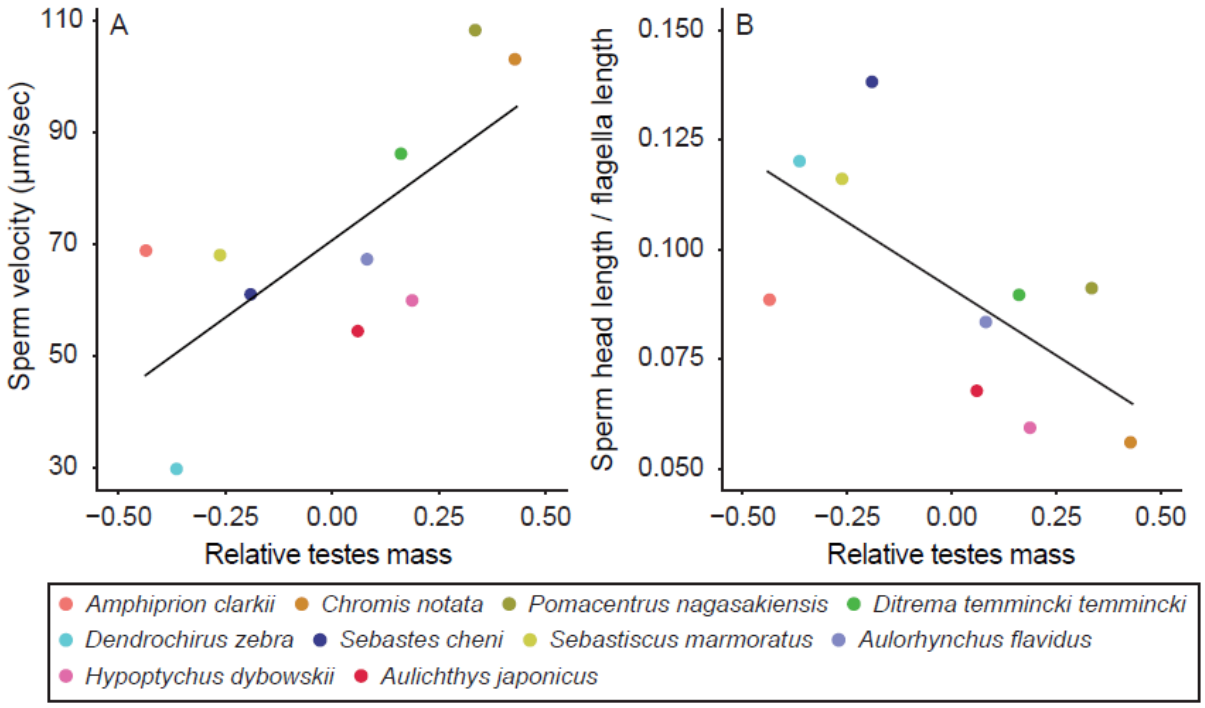

Figure 4

The relationship between relative testes mass (RTM) and sperm components. (A) RTM vs velocity (intercept = 70.74, slope = 55.39). (B) RTM vs the ratio of sperm head length to flagellum length (intercept = 0.091, slope = -0.06). Regression lines are from simple regression analyses (see Table 3 for statistics).

Figure 5

Genital morphology of species with external fertilization and species with internal insemination. (A) Amphiprion clarkii. (B) Chromis notata. (C) Pomacentrus nagasakiensis. (D) Ditrema temmincki temmincki. (E) Dendrochirus zebra. (F) Sebastes cheni. (G) Sebastiscus marmoratus. (H) Aulorhynchus flavidus. (I) Aulichthys japonicus. Scale bars show 5 mm. The arrows indicate the positions of intromittent organs.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1TableS1.docx
- Additionalfile2TableS2.xlsx
- Additionalfile3TableS3.xlsx
- Additionalfile4TableS4.docx
- Additionalfile5TableS5.docx
- Additionalfile6TableS6.docx
- Additionalfile7FigS1.pdf
- Additionalfile8FigS2.pdf