Fertilization modes and the evolution of sperm characteristics in marine fishes: Paired comparisons of externally and internally fertilizing species

Takeshi Ito1,2 | Masaya Morita3 | Seiya Okuno1,2 | Kazuo Inaba4 | Kogiku Shiba4 | Hiroyuki Munehara5 | Yasunori Koya6 | Mitsuo Homma7 | Satoshi Awata1,2

1Department of Biology, Graduate School of Science, Osaka Metropolitan University, Osaka, Japan
2Department of Biology and Geosciences, Graduate School of Science, Osaka City University, Osaka, Japan
3Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Motobu, Japan
4Shimoda Marine Research Center, University of Tsukuba, Shimoda, Japan
5Usujiri Fisheries Station, Field Science Center for Northern Biosphere, Hokkaido University, Hakodate, Japan
6Department of Biology, Faculty of Education, Gifu University, Gifu, Japan
7Diving Service F. WAVE, Sado, Japan

Abstract
Fertilization mode may affect sperm characteristics, such as morphology, velocity, and motility. However, there is little information on how fertilization mode affects sperm evolution because several factors (e.g., sperm competition) are intricately intertwined when phylogenetically distant species are compared. Here, we investigated sperm characteristics by comparing seven externally and four internally fertilizing marine fishes from three different groups containing close relatives, considering sperm competition levels. The sperm head was significantly slenderer in internal fertilizers than in external fertilizers, suggesting that a slender head is advantageous for swimming in viscous ovarian fluid or in narrow spaces of the ovary. In addition, sperm motility differed between external and internal fertilizers; sperm of external fertilizers were only motile in seawater, whereas sperm of internal fertilizers were only motile in an isotonic solution. These results suggest that sperm motility was adapted according to fertilization mode. By contrast, total sperm length and sperm velocity were not associated with fertilization mode, perhaps because of the different levels of sperm competition. Relative testis mass (an index of sperm competition level) was positively correlated with sperm velocity and negatively correlated with the ratio of sperm head length to total sperm length. These findings suggest that species with higher levels of sperm competition have faster sperm with longer flagella relative to the head length. These results contradict the previous assumption that the evolution of internal fertilization increases the total sperm length. In addition, copulatory behavior with internal insemination may involve a large genital morphology, but this is not essential in fish, suggesting the existence of various sperm transfer methods. Although the power of our analyses is not strong because of the limited number of species, we propose a new scenario of sperm evolution in which internal fertilization would increase sperm head length, but not total sperm length, and change sperm motility.
INTRODUCTION

Spermatozoa exhibit a high degree of variation among animal species with respect to morphology and velocity (Pitnick et al., 2009). Several factors have been suggested to contribute to this variation, such as fertilization mode (i.e., external or internal fertilization; Jamieson, 1991; Mohri et al., 2006), phylogeny (Jamieson, 1987), and postulating sexual selection, including sperm competition (Parker, 1970), and cryptic female choice (Pitnick et al., 2009). Fertilization modes and sperm competition are thought to be evolutionary forces that generate sperm diversity (Immler et al., 2007; Jamieson, 1991; Zeng et al., 2014); however, these factors are confounded when phylogenetically distant species are compared, and the relationship between them and sperm characteristics is obscure in many cases (Lüpold & Pitnick, 2018; Pitnick et al., 2009; Simpson et al., 2014).

Generally, the sperm of internally fertilizing species is believed to be longer than that of externally fertilizing species (Franzén, 1970; Lüpold & Pitnick, 2018; Stockley et al., 1996). A recent study also demonstrated that the fertilization mode may drive sperm length evolution with controlled phylogenetic relationships, both across the animal kingdom and at the phylum and class level (Kahrli et al., 2021). This study discusses differences in sperm size with respect to the raffle and displacement mechanisms of sperm competition related to different body sizes. In addition to explaining these mechanisms by body size, considering the effect of actual sperm competition levels on sperm will provide further insight into sperm evolution through the evolution of fertilization mode. The comparison of larger time scales may also miss local evolutionary directions and may involve various confounding factors, even if phylogeny is taken into account. To consider factors, such as fertilization mode and sperm competition level, it is advisable to compare them in closely related species (Cox & Logan, 2021). However, few taxa contain both externally and internally fertilizing species in closely related species, and the evidence that fertilization modes affect sperm characteristics is still limited by not taking into account fertilization mode and sperm competition levels at the same time (Birkhead et al., 2009; Lüpold & Pitnick, 2018).

Sperm competition is also one of the factors that lead to sperm evolution. Over the past 50 years, many studies on sperm competition have documented that sperm competition affects sperm morphology and velocity, both within and across species (Lüpold et al., 2020; Pitnick et al., 2009; Pizzari & Parker, 2009; Simmons & Wedell, 2020). Comparative studies across taxa have shown that species with a higher risk of sperm competition produce longer and faster-swimming sperm (e.g., fishes: Fitzpatrick et al., 2009; mammals: Tourmente et al., 2011; birds: Briskie & Montgomerie, 1992; Immler et al., 2011; Lüpold et al., 2009). By contrast, a number of studies have also shown that sperm competition levels are negatively correlated or uncorrelated with sperm length and velocity (Gage et al., 2002; Gage & Freckleton, 2003; Langen et al., 2019; Stockley et al., 1997). These exceptions are taxon-specific, suggesting that other factors may be relevant. To understand the relationship between sperm evolution and different fertilization modes, it is necessary to consider sperm competition levels, which have a significant effect on sperm traits.

For examining sperm evolution in relation to fertilization modes, fish can be a good model because they exhibit different fertilization modes and various levels of sperm competition, although cartilaginous fish are not suitable because they all perform internal fertilization (Heinicke et al., 2009; Long et al., 2009). Although most teleost species exhibit external fertilization, a few taxa (3%–5% of teleost species) perform internal fertilization with copulatory behavior (Benun Sutton & Wilson, 2019; Fitzpatrick, 2020), which may have evolved multiple times (Goodwin et al., 2002; Reynolds et al., 2002). In addition to normal internal fertilization, several teleosts possess an unusual fertilization mode known as an internal gametic association (IGA) with copulation (Munehara et al., 1989). Only a few teleost fish families exhibit IGA (Evans & Meisner, 2009), including some Aulorhynchidae (Akagawa et al., 2008; Okiyama et al., 1993), Characidae (Pecio et al., 2005), Cottidae (Munehara et al., 1989), and Siluriformes (Parreira et al., 2009). The process by which sperm reaches the egg during IGA is similar to that of internal fertilization (see Section 2). Although species with internal fertilization and IGA are not the majority of teleost fishes, it is possible to compare different fertilization modes (i.e., external vs. internal/IGA) in closely related species.

Previous studies indicated that teleosts with internal fertilization tend to possess elongated sperm heads (i.e., slender heads) and longer sperm than species with external fertilization (Jamieson, 1991; Stockley et al., 1996). However, these studies focused only on the fertilization mode, and sperm competition level was not considered. Similar to internal fertilizing species, the sperm head of IGA species is longer than that of species with external fertilization in marine Cottidae (Baccetti, 1986; Koya et al., 2011; Petersen et al., 2005). However, contrary to the results of these studies, several internally fertilizing fish have oval-headed sperm (Grier et al., 1978; Ito et al., 2021; Yao et al., 1995), and several externally fertilizing fish have sperm with a slender head (Biagi et al., 2016; Hara & Okiyama, 1998; Mattei, 1991). Therefore, the evolutionary effect of the fertilization mode on sperm characteristics remains unclear, even in teleost fish.
Furthermore, teleosts have various mating systems and a wide range of sperm competition levels (Stockley et al., 1997). As the level of sperm competition increases, total sperm length and velocity increase in Tanganyikan cichlid fish (Balshine et al., 2001; Fitzpatrick et al., 2009). 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 (Fitzpatrick, 2020). Therefore, correctly estimating the level of sperm competition allows for the comparison of sperm from external and internal fertilizing species.

Phylogenetic comparative studies within a family or genus are appropriate for considering phylogenetic effects (John & Nicholas, 1997; Paradis, 2014). However, in addition to the small number of internal fertilizers in teleosts, there is sometimes a lack of information on internal fertilizers and their closely related external fertilizing species for which sperm competition levels can be estimated. Hence, it may be difficult to perform large-scale phylogenetic comparisons of fertilization modes considering the level of sperm competition. When a sufficient number of samples cannot be obtained because of ecological factors, paired comparisons may be of use (Balshine et al., 2001). In this case, comparing phylogenetically close species and considering overarching information (e.g., ecology and behavior) could effectively infer specific agents of selection that drive adaptation, and complement even improve on large-scale phylogenetic comparisons (Cox & Logan, 2021).

Here, we investigated the effect of fertilization mode on sperm characteristics in marine fishes, taking into account the different mating systems and sperm competition levels. We chose three groups of marine fishes containing external and internal fertilizing species in close relatives, for which the levels of sperm competition can be inferred from the reproductive system (Group I: Pomacentridae vs. Embiotocidae, Group II: Synanceiidae vs. Sebastinae vs. Scorpaeninae, and Group III: Aulorhynchidae vs. Hypoptychidae; Figure 1). We analyzed the relationship between the fertilization mode or sperm competition, and sperm characteristics (morphology, velocity, and motility in different solutions). We also examined male genital morphology to determine its relationship with fertilization mode, as the genitalia are typically projected in internally fertilizing species (e.g., mammals, cartilaginous fish, and reptiles). These characteristics were analyzed in paired comparisons within groups of closely related species to control for phylogenetic effects and in phylogenetic comparative methods.

2 | MATERIALS AND METHODS

2.1 | Fish sampling and measurements of genital length and testes mass

Focusing on species that can be collected in the seas around Japan, we chose seven species of externally fertilizing fish, three internally fertilizing species, and one IGA species, for which the level of sperm competition could be estimated. They are closely related to each other within the group (see below). In IGA species, males transfer sperm into the ovaries of females by copulation, and sperm swim in the ovarian
fluid and come into contact with eggs (Koya et al., 2002; Munehara et al., 1989). Sperm-egg fusion occurs after the eggs are released into seawater. The fertilization environment is external; however, the process leading up to fertilization is similar to that of internal fertilization. We considered that the selection pressure of IGA on sperm characteristics is almost the same as that of internal fertilization because sperm is transferred to the ovary in the same manner and should adapt to the internal environment. Therefore, we treated the IGA species as an internal fertilizer for the analysis. These 11 species were assigned to three closely related groups (Groups I, II, and III), based on their phylogenetic relationships (Table 1, Figure 1; Kawahara et al., 2009; Near et al., 2013; Nelson et al., 2016; Smith et al., 2018). Group I consisted of externally fertilizing anemonefish (*Amphiprion clarkii*) and damselfish (*Chromis notata* and *Pomacentrus nagasakiiensis*) and internally fertilizing surfperch (*Ditrema temmincki temmincki*). Group II comprised externally fertilizing lionfish (*Dendrochirus zebra*) and waspfish (*Paracentropogon rubripinnis*) and internally fertilizing rockfish (*Sebastes cheni* and *Sebastiscus marmoratus*). Group III consisted of externally fertilizing tube snouts (*Aulichthys flavus*), sand eels (*Hypoppychus dybowskii*), and Japanese tube snouts (*Aulichthys japonicus*), which show IGA (Akagawa et al., 2008; Okiyama et al., 1993). We compared the sperm of different fertilization modes within groups of closely related species, but several pairs were compared between families because there are few families in which both fertilization modes are represented (Table 1).

As data from disparate sources may differ in quality and may be erroneous (Freckleton, 2009), we caught all fish in the field, except for *A. flavus*, and used them for the following analyses. Fish were collected by hand nets using SCUBA, hook and line, and gillnets during the reproductive season in the nearshore waters of Japan and off the west coast of California, USA (Table S1). Three *A. flavus* were obtained from an aquarium in Tokyo Sea Life Park, Japan because we could not access the natural habitat, off the Pacific coast of North America, owing to the COVID-19 pandemic. Note that they were bred in the tank, but the tank was close to natural conditions. The specimens were anesthetized using MS-222 (200 mg/L) and euthanized through cervical transection by snipping the spinal cord between cervical vertebrae 1 and 2 with sharp scissors, according to the American Veterinary Medical Association. Total length (mm), standard length (mm), and body mass (g) were measured. We photographed the genital papilla by gently pressing the abdomen to observe 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). Testes or ovaries of the specimens were removed and weighed (g).

### 2.2 Sperm motility and velocity

The extracted testes were placed on ice in a Petri dish to prevent contamination with urine, mucus, and seawater. Sperm were collected by incising the posterior region of the testes, which contained mature sperm prior to ejaculation, and were used for subsequent analysis. We immediately diluted the extracted semen (<1 μl) in natural seawater or isotonic solution (30μl) to simulate the osmotic pressure of ovarian fluid (150mM NaCl and 10mM HEPES, pH 8.0; Ito & Awata, 2019; Koya et al., 1993) on glass slides coated with 1% bovine serum albumin. Although it is not known if the isotonic solution was the best solution for all species, at least sperm were motile normally (i.e., neither unnatural flagellar movements nor apparently strange head vibrations were observed). Sperm motility was observed in both seawater and isotonic solution using a phase-contrast microscope (DSM-IIIH-104, Daiko Science) equipped with a digital CCD camera (MTV-63WIN, Mintron Enterprise), and the sperm motility was recorded using the Blackmagic Video Recorder software ver. 4.8 (AmScope). One female out of 10 individuals in internally fertilizing D. temmincki temmincki and one female out of seven individuals in A. japonicus were used for sperm measurements (Table S2) using the same method as above because ovarian fluid contained sperm.

Although the effect of ovarian fluid on sperm has not been studied in these two fish species, all sperm swimming speed from the ovary were within the range of that from the testes (see Tables S2, S4, and S6).

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

### 2.3 Sperm morphology

Sperm collected from the posterior region of the testes or ovarian fluid was fixed with optimal fixative (2.5% glutaraldehyde, 0.45M glucose, 60mM HEPES; Ito & Awata, 2019). The sample was placed on a microscope slide, and images of sperm morphology were obtained using a differential interference microscope (BX50, Olympus) equipped with a digital color CCD camera (DMK 33UX174, The Imaging Source) and IC capture software ver. 2.4 (The Imaging Source), or a differential interference microscope (BX53, Olympus), equipped with a digital color CCD camera (DP73, Olympus) and CellSens Standard software ver. 1.9 (Olympus). We measured the morphology of the 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) in the images using ImageJ ver. 1.50i. The head length/width and midpiece length/width ratios were used to determine head and midpiece morphology.

### 2.4 Estimation of sperm competition levels

To increase the resolution of this study, two approaches were applied to estimate sperm competition levels. First, the degree of sperm competition was estimated based on the situational and
| Group | Family (subfamily) | Species | Fertilization mode | Mating system | Sneaker | Density of mating site | Frequency of multiple mating | ESCL | RTM (number of individuals) | References |
|-------|-------------------|---------|--------------------|---------------|---------|------------------------|----------------------------|------|---------------------------|------------|
| I     | Pomacentridae     | *Amphiprion clarkii* | External | Monogamy | Absent | Low | Low | Low | −0.49 ± 0.05 (7) | Moyer and Bell (1976), Moyer and Nakazono (1978) and Ochi (1989) |
|       |                   | *Chromis notata* | External | MTV polygamy | Present? | High | Low | Medium | 0.29 ± 0.28 (20) | Ochi (1985, 1986) and Picciulin et al. (2004) |
|       |                   | *Pomacentrus nagasakiiensis* | External | MTV polygamy | Present? | High | Low | Medium | 0.37 ± 0.1 (8) | Moyer (1975) |
|       | Embiotocidae      | *Ditrema temmincki temmincki* | Internal | MTV polygamy | Present? | High | High | High | −0.07 ± 0.31 (15) | Izumiyama et al. (2020), Nakazono and January (1981) and Takagi et al. (2008) |
| II    | Scorpaenidae      | *Dendrochirus zebra* | External | Promiscuity | Absent | Low | Low | Low | −0.5 ± 0.12 (10) | Moyer (1981) |
|       | (Scopraeninae)    | *Paracentropogon rubripinnis* | External | Promiscuity | Present | High | High | High | 0.49 ± 0.13 (10) | Matsuoka (2005) |
|       | Synanceiidae      | *Sebastes cheni* | Internal | MTV polygamy | Present | Medium | Medium? | Medium | −0.29 ± 0.36 (7) | Blanco Gonzalez et al. (2009), Coleman and Jones (2011), Shinomiya and Ezaki (1991) and Sogard et al. (2008) |
|       | (Sebastinae)      | *Sebastes marmoratus* | Internal | MTV polygamy (Promiscuity) | Absent | Low | Low | Low | −0.36 ± 0.13 (5) | Fujita and Kohda (1996) and Ng et al. (2003) |
| III   | Aulorhynchidae    | *Aulorhynchus flavidus* | External | MTV polygamy | Absent | High | No data | Medium | 0.23 ± 0.15 (4) | Limbaugh (1962) and Schram and Allen (2014) |
|       | Hypoptichidae     | *Hypopterus dybowskii* | External | MTV polygamy | Present | High | High | High | 0.19 ± 0.08 (6) | Akagawa and Okiyama (1993) and Narimatsu and Munehara (2001) |
|       | Aulorhynchidae    | *Aulichthys japonicus* | IGA | MTV polygamy | Absent | High | Medium | Medium | 0.06 ± 0.11 | Akagawa et al. (2008, 2004) and Okiyama et al. (1993) |

Abbreviation: IGA, Internal gametic association; MTV polygamy, Male-territory-visited polygamy.

*The mating system of *D. zebra* has been reported to be promiscuous, but mating occurs between dyadic relationships (i.e., monogamous). Therefore, the level of sperm competition was predicted to be low.

*Closedly related species *S. inermis* and *S. atrovirens* showed male-territory-visited polygamy and multiple paternities.

*Aquarium observations revealed the occurrence of sneaker males (Takamizo et al., 2015), but we referred to field studies to arrange the conditions among species, as other studies were conducted in the field.*
relationships in these trees were expected to be similar to those in trees based on the original species because each substitute species represents the same family or genus as the original species. Therefore, we merged the divergence time between *S. marmoratus* and *Sebastes cheni* of the trimmed tree of Rabosky et al. (2013) with that of Betancur et al. (2017) and used it for further analyses (the merged tree is shown in Figure S1).

### 2.6 | Data analyses

Although we collected mature sperm from the posterior region of the testis, to rule out the possibility of measuring immature sperm, we eliminated outliers from the datasets using the IBM SPSS Statistics ver. 23.0 (SPSS, Inc.) before analyses (50 out of 1262 [3.9%] sperm for morphology and 40 out of 1689 sperm [2.4%] for velocity, Tables S4–S6). We compared sperm characteristics, including total sperm length, head length and width, midpiece length and width, and velocity among external and internal fertilizers within the same group (Groups I, II, or III). Analyses were conducted on three groups using linear mixed models (LMMs) as implemented in the “lme4” package (Bates et al., 2015) with R 3.4.1 (R Core Team, 2016). In the models, we set “species” as a fixed effect and “individual ID” as a random effect because we measured sperm characteristics of multiple sperm per individual. Multiple comparisons among species were conducted using Tukey’s all-pair comparisons with the “multcomp” package (Hothorn et al., 2016) to control family-wise error rates among species. Differences were considered statistically significant at *p* < .05.

In addition to the LMM analyses, we also performed a phylogenetic generalized least squares (PGLS) approach (Freckleton et al., 2002; Pagel, 1999) to improve the resolution of the research and to reveal the relationship between fertilization mode or sperm competition and sperm characteristics in a phylogenetic context. Although our sample size was small, a model with a few predictors (two or three) might still reveal reasonable results with a considerably small dataset (Mundry, 2014), and our models focused on two predictors. We used fertilization mode and sperm competition levels as independent variables in each model. An interaction term between independent variables was not incorporated into the model, as our dataset was not sufficiently large. PGLS analyses were conducted using the R package “caper” (Orme et al., 2013) in a maximum-likelihood framework. To assess the phylogenetic nonindependence of the averaged species data, Pagel’s lambda was estimated according to the ML estimate (0: low phylogenetic signal; 1: high phylogenetic signal), although the lambda estimates were less robust when the sample size was <20 species (Freckleton et al., 2002).

For the index of sperm competition, we calculated the residuals of all individuals as RTM from the regression line estimated by the LMM based on the relationship between log(10) soma mass (body mass–visceral mass–testes mass) and log(10) testes mass, setting
species as a random effect (Figure S2). We performed a PGLS regression analysis between RTM and sperm characteristics to assess whether the species in which males invested more in the testes would have superior sperm (i.e., faster-swimming sperm). We also examined the relationship between sperm velocity and morphological components using the PGLS approach.

To assess whether males of internally fertilizing species had elongated genitalia, we calculated the relative genital length. The relative genital length was estimated as the residual from the regression line estimated by an LMM on the relationship between log_{10} standard length (mm) and log_{10} genital length (mm), and the species ID was set as a random effect (Figure S3). The mean relative genital length was compared using ANOVA, followed by the Tukey’s HSD test for multiple comparisons. We also examined whether relative genital length was related to fertilization mode and sperm competition level using the PGLS approach.

3 | RESULTS

3.1 | Sperm morphology

Sperm morphology was diverse among species with external and internal fertilization, including IGA (Figure 2). In Group I, the total sperm and flagella lengths of internally fertilizing species with higher estimated sperm competition levels were significantly longer than those of externally fertilizing species (Figure 3A, Table S4). However, there was no apparent relationship between fertilization mode and total sperm length or flagella length in Groups II and III. Significant differences in total sperm length and flagella length were detected in Groups II (Figure 3B, Table S5) and III (Figure 3C, Table S6), and these traits seemed to be related to sperm competition. PGLS analyses also showed that the total sperm length and flagella length increased evolutionarily with increasing levels of sperm competition (Table 2).

Sperm head morphology differed between externally and internally fertilizing/IGA species in all the groups (Figure 2). Sperm heads of internal fertilizers/IGA species were more slender than those of external fertilizers, which showed spherical or oval morphology (Figure 3D–F; Tables S4–S6). One exception was Hypoptychus dybowskii in Group III (Figure 3F, Table S6), which had a slightly longer sperm head than the other two externally fertilizing species. However, no statistical difference in the head ratio was found between A. japonicus and H. dybowskii. PGLS analysis showed that head morphology was elongated by the evolution of fertilization modes from external to internal, and was not related to sperm competition (Table 2).

The ratio of midpiece length to width showed similar results to head morphology, in which internally fertilizing/IGA species had a longer midpiece than externally fertilizing species in groups I and III (Figure 3G, Tables S4 and S6). Nevertheless, there was no trend between midpiece morphology and fertilization mode or sperm competition in Group II (Figure 3H, Table S5). The PGLS analysis also showed a (nonsignificant) trend of a positive evolutionary relationship between the midpiece ratio and fertilization mode (Table 2).
IT \ et \ al.

Group I

**Total sperm length (μm)**

| Group | Ac | Cn | Pr | Sc | Sm | Dtt |
|-------|----|----|----|----|----|-----|
| I     | a  | b  | c  | a  | b  | d   |
| II    | a  | b  | c  | c  | a  | b   |
| III   | a  | b  | c  | a  | b  | c   |

**Head morphology (head length / head width)**

| Group | Ac | Cn | Pr | Sc | Sm | Dtt |
|-------|----|----|----|----|----|-----|
| I     | a  | b  | a  | a  | c  | b   |
| II    | b  | b  | a  | a  | b  | c   |
| III   | a  | b  | a  | b  | c  | a   |

**Midpiece morphology (midpiece length / midpiece width)**

| Group | Ac | Cn | Pr | Sc | Sm | Dtt |
|-------|----|----|----|----|----|-----|
| I     | a  | b  | a  | b  | a  | c   |
| II    | a  | b  | a  | b  | a  | c   |
| III   | a  | b  | a  | b  | a  | c   |

**Sperm velocity (μm/sec)**

| Group | Ac | Cn | Pr | Sc | Sm | Dtt |
|-------|----|----|----|----|----|-----|
| I     | a  | b  | c  | c  | c  | b   |
| II    | a  | b  | c  | c  | c  | b   |
| III   | a  | b  | c  | c  | c  | b   |

**Legend**

- *LESC: MM* (LESC: Median, Median)
- *HM HM* (High, Median, High, Median)
FIGURE 3 Differences in sperm morphological characteristics and sperm velocity (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 velocity in Group I (j), Group II (k), and Group III (l). White bars and black bars indicate species with external fertilization and internal insemination, respectively. Estimated relative sperm competition levels (ESCL) are also shown under the bar: Low (L), medium (M), and high (H).

Ac, Amphiprion clarkii; Af, Aulorhynchus flavidus; Aj, Aulichthys japonicus; Cn, Chromis notata; Dtt, Ditrema temmincki temmincki; Dz, Dendrochirus zebra; Hd, Hypoptychus dybowskii; Pn, Pomacentrus nagasakiensis; Pr, Paracentropogon rubripinnis; Sc, Sebastes cheni; Sm, Sebastiscus marmoratus. Different letters indicate significant differences between species (LMMs with sequential Bonferroni correction, p < .05).

### TABLE 2

| Traits                      | λ     | 95% CI  | Adjusted R² | Predictors | F     | p   |
|-----------------------------|-------|---------|--------------|------------|-------|-----|
| Total sperm length (µm)     | 0.01  | NA–0.67 | .60          | FM         | 0.02  | .89 |
| Flagella length (µm)        | 0.01  | NA–0.70 | .59          | FM         | 0.13  | .73 |
| Head length (µm)            | 0.004 | NA–0.60 | .13          | FM         | 4.05  | .08 |
| Head width (µm)             | 0.03  | 0.33–NA | .49          | FM         | 6.27  | .04 |
| Midpiece length (µm)        | 0.09  | NA–NA   | .22          | FM         | 5.29  | .06 |
| Midpiece width (µm)         | 0.003 | NA–0.65 | .15          | FM         | 0.06  | .82 |
| Head length/head width      | 0.14  | NA–NA   | .59          | FM         | 11.36 | .01 |
| Midpiece length/midpiece width | 0.09 | NA–NA   | .18          | FM         | 3.90  | .09 |
| Sperm velocity (µm/s)       | 0.30  | NA–NA   | .03          | FM         | 0.83  | .39 |

Note: Superscripts following the λ estimates represent the significance levels of the likelihood-ratio tests (first position: against λ = 0; second position: against λ = 1). The adjusted R² and 95% confidence intervals (CIs) were calculated for each model. Bold prints indicate statistically significant correlations.

### TABLE 3

| 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          |
|       | Paracentropogon rubripinnis | External       | Motile                   | Immotile          |
|       | Sebastiscus cheni        | Internal           | Immotile                 | Motile            |
|       | Sebastiscus marmoratus   | Internal           | Immotile                 | Motile            |
| III   | Aulorhynchus flavidus    | External           | Motile                   | Motile            |
|       | Hypoptychus dybowskii    | External           | Motile                   | Immotile          |
|       | Aulichthys japonicus     | IGA                | Immotile                 | Motile            |

Abbreviation: IGA, Internal gametic association.
Sperm motilities in different solutions differed between external and internal fertilizers/IGA in all three groups (Table 3). The sperm of externally fertilizing species were motile in seawater but immotile in an isotonic solution that imitated ovarian fluid. By contrast, sperm of internally fertilizing species, including IGA species, were motile in isotonic solution but immotile in seawater. Only the sperm of *Aulorhynchus flavidus* was active in both seawater and isotonic solutions (Table 3).

We did not obtain consistent results between sperm velocity and fertilization mode (Figure 3; Tables S4–S6). Regarding sperm competition, in Group I, the sperm velocity of *A. clarkii*, in which the sperm competition level was low, was lower than that of other species (Figure 3J, Table S4). In Group II, *P. rubripinnis*, facing higher levels of sperm competition, produced markedly faster sperm than the other species with low or medium levels of sperm competition (Figure 3K, Table S5). However, no trend between sperm velocity and sperm competition level was observed in Group III; *A. flavidus* had faster sperm than the other two species (Figure 3L, Table S6). No relationship was found between sperm velocity and fertilization mode or sperm competition levels using the PGLS approach (Table 2).

**TABLE 4** Relationships between sperm characteristics and relative testes mass (RTM) and between sperm velocity and sperm morphological components

| Characteristic                  | Predictor | 95% CI   | Estimate ± SE | Multiple $R^2$ | $F$    | $\lambda$ | $p$         |
|---------------------------------|-----------|----------|---------------|----------------|--------|-----------|-------------|
| Total sperm length              | RTM       | NA–NA    | 12.40 ± 6.97  | .26            | 3.17   | 0.01      | .14         |
| Flagella length                 | RTM       | NA–NA    | 13.25 ± 6.72  | .30            | 3.89   | 0.01      | .13         |
| Head length                     | RTM       | NA-0.91  | −0.84 ± 0.51  | .23            | 2.71   | 0.01      | .02         |
| Head width                      | RTM       | NA-0.39  | 0.07          |                | 0.70   | 1.0       | .06, 1.0    |
| Midpiece length                 | RTM       | NA-0.53  | 0.08          |                | 0.08   | 1.0       | .14, .79    |
| Midpiece width                  | RTM       | NA-0.32  | .42           | 6.54           | 0.04   | 0.92, 0.22| .03         |
| Head length/head width          | RTM       | NA-0.48  | 0.05          | .05            | 0.97   | 0.92, 0.22| .03         |
| Midpiece length/midpiece width  | RTM       | NA-0.48  | .02           | 0.19           | 1.0    | 0.05, 1.0 | .67         |
| Head length/total sperm length  | RTM       | NA-0.01  | −0.05 ± 0.01  | .59            | 12.97  | 0.01      | .02         |
| Sperm velocity                  | RTM       | NA-NA    | 82.49 ± 27.46 | .50            | 9.03   | 1.0       | .28, 1.0    |
| Total sperm length              | RTM       | NA-NA    | 3.61 ± 1.53   | .38            | 5.55   | 0.01      | .15         |
| Head length/Total sperm length  | RTM       | NA-NA    | −1142 ± 592   | .29            | 3.72   | 0.01      | .37         |
| Head length                     | RTM       | NA-NA    | −16.05 ± 26.55| .04            | 0.37   | 0.01      | .75         |
| Midpiece length                 | RTM       | NA-NA    | −4.30 ± 21.64 | .004           | 0.04   | 0.01      | .11         |

Note: Bold print indicates statistically significant correlations.

### 3.2 Sperm motility and velocity

Sperm motilities in different solutions differed between external and internal fertilizers/IGA in all three groups (Table 3). The sperm of externally fertilizing species were motile in seawater but immotile in an isotonic solution that imitated ovarian fluid. By contrast, sperm of internally fertilizing species, including IGA species, were motile in isotonic solution but immotile in seawater. Only the sperm of *Aulorhynchus flavidus* was active in both seawater and isotonic solutions (Table 3).

**FIGURE 4** Relationship between relative testes mass (RTM) and sperm components. (a) Correlation between RTM and the ratio of sperm head length to total sperm length (PGLS, intercept = 0.08, slope = −0.061). (b) Correlation between RTM and velocity (PGLS, intercept = 82.49, slope = 92.97).

Regression lines are from PGLS analyses (see Table 4 for statistics).

### 3.3 Relationships between RTM and sperm characteristics

Phylogenetic generalized least squares analysis revealed that the ratio of sperm head length to total sperm length was negatively correlated with RTM (Figure 4A, Table 4), whereas sperm velocity was positively correlated with RTM (Figure 4B). No correlation was
detected for the other sperm components, except for the midpiece width (Table 4). We also found that total sperm length was positively correlated with sperm velocity, although this relationship was no longer significant when the outlier (P. rubripinnis) was eliminated (PGLS; $F = 0.02, p = .90$).

3.4 | Genital morphology

As expected, six of the seven external fertilizers did not have apparent genitalia (Figure 5A,B,E,F,I,J, Table 5). Nevertheless, a relatively sizeable genital papilla appeared in P. nagasakii after pressing
the abdomen (Figure 5C). Of the internal fertilizers and species with IGA, *S. marmoratus* and *A. japonicus* had large and slender genitalia that were retracted in the abdominal cavity unless the abdomen was pressed (Figure 5H,K). The other internally fertilizing fishes, such as *D. temmincki temmincki* and *S. cheni*, had no noticeable copulatory organs, although a small genital papilla was present (Figure 5D,G). Although significant differences in relative genital length were detected between species in all three groups (Table 5), the PGLS approach showed that neither fertilization mode (*F* = 2.02, *p* = .20) nor sperm competition level (*F* = 0.31, *p* = .75) affected the relative genital length (*i* = 0.10.02, 95% CI = NA–0.764).

### 4 | DISCUSSION

Previous studies on teleost fish have suggested that internal fertilizers, including IGA species, have sperm with a more elongated head than external fertilizers (Jamieson, 1991; Koya et al., 2011). Notably, our results suggest the same tendency as observed in previous studies, even when close relatives were compared, and phylogeny was controlled. Thus, different fertilization patterns could be a strong evolutionary force affecting the sperm head, and we demonstrated the effect of fertilization modes on sperm head morphology. Sperm head morphology affects sperm swimming behavior (Muto & Kubota, 2009; Støstad et al., 2018). This is probably because a narrow head reduces drag so that slender-headed sperm in internal fertilizing/IGA species can travel easily through the viscous ovarian fluid (Malo et al., 2006; Tourmente et al., 2011; Watanabe et al., 2000). In addition, the viscosity of the ovarian fluid is 2 to 3-fold higher than that of water (Zadmadj et al., 2019). 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 that of the spherical morphology (i.e., 1:1 ratio), although a more slender head (ratio exceeding 4:1) increases the drag more than a sphere (Humphries et al., 2008). In this study, the head ratios of the internal fertilizers were 1.29–2.83 (see Tables S4–S6). The hypothesis that the sperm head of a species undergoing internal insemination is elongated to adapt to a viscous environment is likely applicable to the fish used in the current study. Generally, the ovarian lumen has a somewhat complex structure and is narrow; sperm motility may be more obstructed in the ovary than in seawater, which is an unobstructed space. Therefore, an elongated head may be more suitable for propulsion in structurally complex ovarian environments.

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 Gasterosteoidei (Hara et al., 2013; Hara & Okiyama, 1998). The selective pressure of the viscous ovarian fluid on sperm may also be expected to act on species with external fertilization because spawned eggs are covered by viscous ovarian fluid (Zadmadj et al., 2019). The elongated head in externally fertilizing *H. dybowskii* may also adapt to the viscous fluid, although externally fertilizing *A. flavidus* with an oval head have a reproductive mode similar to that of *H. dybowskii* (Akagawa & Okiyama, 1993; Limbaugh, 1962). In Baikal sculpins, the sperm of externally and internally fertilizing species also have the same head morphology, whereas the head volume is smaller in internally fertilizing than in externally fertilizing species, which may be an adaptation to viscous environments (Ito et al., 2021). A smaller sperm head has a lower drag force against a solution than a larger head (Marcos et al., 2014). In the present study, there was no difference in the cross-sectional area of the head between *H. dybowskii* and *A. japonicus* (Table S6). The reason why sperm head morphology was similar between *H. dybowskii* and *A. japonicus* remains unknown, and further detailed research is required to clarify this issue.

Although the sample size was limited in this study, LMM and PGLS analyses support that total sperm length (including flagella length) and velocity varied and may be independent of fertilization mode, in contrast to head morphology in both analyses. Comparative studies among a wide range of taxa have shown that internal fertilizers tend to have longer sperm than external fertilizers (Franzén, 1970; Lüpold & Pitnick, 2018). In bony fish, a previous

---

**Table 5** Fertilization mode and relative genital length in male fish

| Group | Species | Fertilization mode | Relative genital length (n) | ANOVA |
|-------|---------|--------------------|-----------------------------|-------|
| I     | *Amphiprion clarkii* | External | -0.35 ± 0.20\(\lambda\) (4) | \(F = 21.80\) |
|       | *Chromis notata* | External | -0.15 ± 0.03\(\lambda\) (3) | \(p < .001\) |
|       | *Pomacentrus nagasakiensis* | External | 0.69 ± 0.13\(\lambda\) (3) | \(p < .001\) |
|       | *Ditrema temmincki temmincki* | Internal | 0.02 ± 0.21\(\lambda\) (5) | \(F = 0.31\) |

II

| Species | Fertilization mode | Relative genital length (n) | ANOVA |
|---------|--------------------|-----------------------------|-------|
| *Dendrochirus zebra* | External | -0.64 ± 0.23\(\lambda\) (2) | \(F = 43.79\) |
| *Paracentropogon rubripinnis* | External | 0.32 ± 0.10\(\lambda\) (8) | \(p < .001\) |
| *Sebastes cheni* | Internal | -0.03 ± 0.13\(\lambda\) (6) | \(F = 735.36\) |
| *Sebastiscus marmoratus* | Internal | 0.24 ± 0.09\(\lambda\) (9) | \(p < .001\) |

III

| Species | Fertilization mode | Relative genital length (n) | ANOVA |
|---------|--------------------|-----------------------------|-------|
| *Aulorhynchus flavidus* | External | -1.02 ± 0.11\(\lambda\) (3) | \(F = 21.80\) |
| *Hypoptychus dybowskii* | External | -0.25 ± 0.17\(\lambda\) (2) | \(p < .001\) |
| *Aulichthys japonicus* | IGA | 1.15 ± 0.02\(\lambda\) (6) | \(p < .001\) |

Note: Different superscripts indicate significant differences according to the Tukey’s HSD test (\(p < .05\)) among species in each group. The number of individuals is shown in parentheses.
study in which phylogeny was partially considered indicated a similar result (Stockley et al., 1996). A recent meta-analysis of a wide range of animals also showed that internal fertilizers have longer sperm than external fertilizers (Kahrl et al., 2021), and there was higher power to detect sperm length differences, although the actual sperm competition levels were not considered. Our comparisons with close relatives and PGLS analysis were weak power owing to sample size but indicated that fertilization mode might not affect sperm length. This result is partially consistent with that of Kahrl et al. (2021); sperm length was not statistically significant in the clade of bony fish, although sperm length was 1.2-fold larger in internal fertilizers than in external fertilizers. We also showed that the fertilization mode and sperm competition level did not influence sperm 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 (Balshine et al., 2001; Fitzpatrick et al., 2009; Immler et al., 2011; Kleven et al., 2009; Tournmente et al., 2011). Our paired comparison results of total sperm length and velocity were partially consistent with these studies; species with a low level of sperm competition produced 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 confirm this assumption (e.g., sperm length: A. clarkii vs. C. notata and P. nagasakiensis; velocity: S. cheni vs. S. marmoratus), and there was no relationship between sperm total length and velocity. Overall, the PGLS analysis showed that total sperm length was correlated with sperm competition level. The long flagellum (but not the extra-long flagellum) is advantageous for swimming speed (Ishijima et al., 1998), such that species with high levels of sperm competition may have longer flagella (Ball & Parker, 1996).

We showed that RTM, a proxy for sperm competition, did not correlate with the total sperm length. However, the ratio of head to total sperm length was negatively correlated with RTM, suggesting that sperm competition promotes relatively longer sperm relative to the head length. The theoretical model predicts that sperm with small heads relative to their total length reduces the drag force to swim in water; thus, relatively longer flagella swim faster (Humphries et al., 2008). The sperm velocity was also positively correlated with RTM. Thus, increasing sperm competition levels may promote a relatively longer flagella-to-head length and faster sperm velocity, and the results are likely to match the theory of sperm competition (Ball & Parker, 1996; Parker, 1993).

There was no apparent relationship between the midpiece morphology and fertilization mode or sperm competition. The midpiece contains mitochondria, which generate energy; thus, midpiece volume is likely to be related to sperm velocity (Anderson & Dixson, 2002; Firman & Simmonds, 2010; Gil et al., 2009; Tournmente et al., 2009, 2011), and midpiece size is positively correlated with different levels of sperm competition (Anderson et al., 2005; Anderson & Dixson, 2002; Tournmente et al., 2009, 2011). Furthermore, one study reported that different tactics (i.e., sneaker and courting) affect sperm midpiece morphology in internally fertilizing Xiphophorus nigrensis (Smith & Ryan, 2010). However, our results showed that midpiece size was not related to the estimation of relative sperm competition levels. Sperm velocity was also not correlated with midpiece size. The ecological and morphological functions of the midpiece were not determined in the present study.

We showed that sperm motility was strongly dependent on the fertilization mode. In the current study, the sperm of externally fertilizing species were motile only in seawater, except for that of A. flavidus, which sperm were motile in both seawater and isotonic solutions. 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 exhibit sperm motility in both seawater/freshwater and isotonic solutions (Hayakawa & Munehara, 1998; Ito et al., 2011; Petersen et al., 2005). Thus, some species with external fertilization may be able to move in the ovarian fluid, generating the foothold of internal fertilization (Ito et al., 2021). By contrast, all sperm of internally fertilizing species, including the IGA species in the current study, progressed only in isotonic solution but not in seawater. Similar results have been reported for marine sculpins with IGA (Abe & Munehara, 2007; Koya et al., 1993). In addition, the sperm of freshwater fish with viviparity were only motile in isotonic solution but immotile in low osmotic solution in Baikal sculpin (Ito et al., 2021) and swordtail (Yang et al., 2006). Therefore, the sperm of the internal inseminator may have adapted to the internal environment. A previous study showed that the sperm of marine fish can swim in media over a comparatively larger range of osmolarity than that of freshwater fish (Alavi & Cosson, 2006). However, little information is available on sperm motility in marine fish subjected to internal fertilization. Based on a previous study on externally fertilizing marine fish (Alavi & Cosson, 2006) and our results, we conclude that the sperm motility of marine fish with internal fertilization and IGA is restricted to an isotonic solution and does not require motility in seawater because of the evolution of fertilization modes.

In the present study, even externally fertilizing P. nagasakiensis had a relatively sizeable genital papilla. A previous field study has shown that the genital papilla is used for ejaculation by protruding to rub eggs during mating (Moyer, 1975). With a few exceptions, such as P. nagasakiensis, externally fertilizing species do not require large genitalia. By contrast, the large genital organ was not associated with internal fertilization, contrary to popular belief that: two internal fertilizers (D. temmincki temmincki and S. cheni) did not have large genitalia for copulation, suggesting that copulatory organs may not be necessary for internal insemination in fish. These results suggest that even internally fertilizing fish may copulate in various ways, not simply using their genitalia, and have evolved a peculiar way of transferring sperm. The sea raven Hemitripterus villosus, which exhibits IGA, lacks large genitalia but ejaculates semen toward the genital duct projecting from a female for copulation (Munehara, 1996). Viviparous eelpouts (Zoaridae) also do not have large genitalia and copulate through direct genital contact, although the details of their...
mating behavior are still unknown (Yao & Crim, 1995). More detailed observations of copulation behavior are necessary to clarify the variation in sperm transfer. Several hypotheses have been proposed regarding the evolution of large genitalia and their diversity, including male–male competition, female choice, and predation risk (Brennan & Prum, 2015; Heinen-Kay & Langerhans, 2013; Langerhans, 2011). However, future work should also focus on animals that do not have genitalia despite performing internal fertilization in order to improve our understanding of the evolution of internal fertilization.

5 | 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 and in phylogenetic comparative methods. Although the sample size was limited and statistical power was not strong, we demonstrated that internal fertilization, including IGA, may influence sperm head morphology and motility in the extracellular environment. Total sperm length (including flagellum length) and velocity may be associated with sperm competition, but not fertilization mode, although there are a few exceptions. Previous studies have suggested that internal fertilizers have longer sperm than external fertilizers (Franzén, 1970; Lüpold & Pitnick, 2018; Stockley et al., 1996); however, our paired comparison and phylogenetic comparative analyses suggest that internal fertilization with internal insemination and IGA may have a little evolutionary effect on total sperm length when comparing closely related species and considering sperm competition levels.

AUTHOR CONTRIBUTIONS

Takeshi Ito: Formal analysis (lead); funding acquisition (equal); investigation (equal); methodology (equal); visualization (lead); writing – original draft (lead). Masaya Morita: Formal analysis (supporting); investigation (supporting); writing – review and editing (equal). Seiya Okuno: Formal analysis (lead); methodology (supporting); writing – review and editing (supporting). Kazuo Inaba: Formal analysis (supporting); writing – review and editing (supporting). Kogiku Shiba: Formal analysis (supporting); writing – review and editing (supporting). Satoshi Awata: Formal analysis (equal); supervision (lead); writing – review and editing (lead).

ACKNOWLEDGMENTS

We thank Nagaaki Sato and Namiko Sato (diving service Grunt Sculpin), Shun Sato, Taichi Yamada (Osaka City University), Yoshihisa Sato (Sado Ageshima Aquarium), Katsunori Tachihiara (University of the Ryukyus), and late Shohei Suzuki (Okinawa Institute of Science and Technology, Graduate University) for their assistance with diving and sampling. We would also like to thank Editage (www.editage.jp) for the English language editing. This study was financed by the Japan Society for the Promotion of Science (JSPS) KAKENHI (Grant No. JP19J11278 to TI, and JP16H04841 and JP17K19518 to SA), JSPS Overseas Challenge Program for Young Researchers (Grant No. 20190182 to TI), Sasakawa Scientific Research Grant from the Japan Science Society (Grant No. 29-541 to TI), Collaborative Research of Tropical Biosphere Research Center, JAMBlO, ABiS, and Osaka University (OCU) Strategic Research Grant 2020 (to SA).

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The datasets used and analyzed in the current study are shown in Tables S2 and S3.

ORCID

Takeshi Ito https://orcid.org/0000-0001-8664-6722
Seiya Okuno https://orcid.org/0000-0001-8923-7108
Kazuo Inaba https://orcid.org/0000-0001-8848-9733
Kogiku Shiba https://orcid.org/0000-0002-7742-8158
Satoshi Awata https://orcid.org/0000-0003-3254-7943

REFERENCES

Abe, T., & Munehara, H. (2007). Histological structure of the male reproductive organs and spermatogenesis in a copulating sculpin, Radulinospis taranetzi (Scorpaeniformes: Cottidae). Ichthyological Research, 54, 137–144.

Akagawa, I., Hara, M., & Iwamoto, T. (2008). Egg concealment in ascidians by females of the Japanese tubesnout, Aulichthys japonicus (Gasterosteiformes), and its subsequent copulation. Ichthyological Research, 55, 85–89.

Akagawa, I., Iwamoto, T., Watanabe, S., & Okiyama, M. (2004). Reproductive behaviour of Japanese tubesnout, Aulichthys japonicus (Gasterosteiformes), in the natural habitat compared with relatives. Environmental Biology of Fishes, 70, 353–361.

Akagawa, I., & Okiyama, M. (1993). Alternative male mating tactics in Hypoctychus dybowskii (Gasterosteiformes): Territoriality, body size and nuptial colouration. Japanese Journal of Ichthyology, 40, 343–350.

Alavi, S. M. H., & Cosson, J. (2006). Sperm motility in fishes. (II) Effects of ions and osmolality: A review. Cell Biology International, 30, 1–14.

Anderson, M. J., & Dixon, A. F. (2002). Sperm competition: Motility and the midpiece in primates. Nature, 416, 496.

Anderson, M. J., Nyholt, J., & Dixon, A. F. (2005). Sperm competition and the evolution of sperm midpiece volume in mammals. Journal of Zoology, 267, 135–142.

Baccetti, B. (1986). Evolutionary trends in sperm structure. Comparative Biochemistry and Physiology Part A: Physiology, 85, 29–36.

Baker, J., Humphries, S., Ferguson-Gow, H., Meade, A., & Venditti, C. (2019). Rapid decreases in relative testes mass among monogamous birds but not in other vertebrates. Ecology Letters, 23, 283–292. https://doi.org/10.1111/ele.13431

Ball, M. A., & Parker, G. A. (1996). Sperm competition games: External fertilization and "adaptive" infertility. Journal of Theoretical Biology, 180, 141–150.

Balshine, S., Leach, B. J., Neat, F., Werner, N. Y., & Montgomery, R. (2001). Sperm size of African cichlids in relation to sperm competition. Behavioral Ecology, 12, 726–731.
Bates, D., Mächler, M., Bolker, B. M., & Walker, S. C. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software, 67*, 1–48.

Benun, Sutton, F., & Wilson, A. B. (2019). Where are all the moms? External fertilization predicts the rise of male parental care in bony fishes. *Evolution, 73*, 2451–2460.

Betancur, R. R., Wiley, E. O., Arratia, G., Acero, A., Bailly, N., Miyra, M., Lecointre, G., & Ortí, G. (2017). Phylogenetic classification of bony fishes. *BMC Evolutionary Biology, 17*, 1–40.

Biagi, F., Piras, F., Farina, V., Zedda, M., Mura, E., Floris, A., Franzoi, P., Fausto, A. M., Taddei, A. R., & Carcupino, M. (2016). Testis structure, spermatogenesis and sperm morphology in pipefishes of the genus *Syngnathus*. *Acta Zoologica, 97*, 90–101.

Birkhead, T. R. (1998). Sperm competition in birds. *Reviews of Reproduction, 3*, 123–129.

Birkhead, T. R., Hosken, D. J., & Pitnick, S. (2009). *Sperm biology: An evolutionary perspective*. Academic Press.

Blanco Gonzalez, E., Murakami, T., Teshima, Y., Yoshioka, K., Jeong, D. S., & Umino, T. (2009). Paternity testing of wild black rockfish *Sebastes inermis* (brownish type) from the Seto Inland Sea of Japan. *Ichthyological Research, 56*, 87–91.

Brennan, P. L. R., & Prum, R. O. (2015). Mechanisms and evidence of genetic coevolution: The roles of natural selection, mate choice, and sexual conflict. *Cold Spring Harbor Perspectives in Biology, 7*, 1–21.

Briskie, J. V., & Montgomerie, R. (1992). Sperm size and sperm competition in birds. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 247*, 89–95.

Coleman, S. W., & Jones, A. G. (2011). Patterns of multiple paternity and maternity in fishes. *Biological Journal of the Linnean Society, 103*, 735–760.

Cox, C. L., & Logan, M. L. (2021). Using integrative biology to infer adaptation from comparisons of two (or a few) species. *Physiological and Biochemical Zoology, 94*, 162–170.

Dunn, P. O., Whittingham, L. A., & Pitcher, T. E. (2001). Mating systems, sperm competition, and the evolution of sexual dimorphism in birds. *Evolution, 55*, 161–175.

Evans, J. P., & Meisner, A. D. (2009). Copulatory structures: Taxonomic overview and the potential for sexual selection. In B. G. M. Jamieson (Ed.), *Reproductive biology and phylogeny of fishes (agnathans and bony fishes)* (Vol. 8B, pp. 138–180). Science Publishers.

 Firman, R. C., & Simmons, L. W. (2010). Sperm midpiece length predicts sperm swimming velocity in house mice. *Biological Letters, 6*, 513–516.

Fitzpatrick, J. L. (2020). Sperm competition and fertilization mode in fishes. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 375*, 20200074.

Fitzpatrick, J. L., Montgomerie, R., Desjardins, J. K., Stiver, K. A., Kolm, N., & Balshine, S. (2009). Female promiscuity promotes the evolution of faster sperm in cichlid fishes. *Proceedings of the National Academy of Sciences of the United States of America, 106*, 1128–1132.

Franzén, Å. (1970). Phylogenetic aspects of the morphology of spermatozoa and spermogenesis. In B. Baccetti (Ed.), *Comparative spermatology* (pp. 29–45). Academic Press.

Freckleton, R. P. (2009). The seven deadly sins of comparative analysis. *Journal of Evolutionary Biology, 22*, 1367–1375.

Freckleton, R. P., Harvey, P. H., & Pagel, M. (2002). Phylogenetic analysis and comparative data: A test and review of evidence. *The American Naturalist, 160*, 712–726.

Fujita, H., & Kohda, M. (1996). Male mating effort in the viviparous scorpionfish, *Sebastiscus marmoratus*. *Ichthyological Research, 43*, 247–255.

Gage, M. J. G., & Freckleton, R. P. (2003). Relative testis size and sperm morphometry across mammals: no evidence for an association between sperm competition and sperm length. *Proceedings of the Royal Society B: Biological Sciences, 270*, 625–632.

Gage, M. J. G., Macfarlane, C., Yeates, S., Shackleton, R., & Parker, G. A. (2002). Relationships between sperm morphometry and sperm motility in the Atlantic salmon. *Journal of Fish Biology, 61*, 1528–1539.

Gil, M. C., García-Herreros, M., Barón, F. J., Aparicio, I. M., Santos, A. J., & García-Marín, L. J. (2009). Morphometry of porcine spermatozoa and its functional significance in relation with the motility parameters in fresh semen. *Theriogenology, 71*, 254–263.

Goodwin, N. B., Dulvy, N. K., & Reynolds, J. D. (2002). Life-history correlates of the evolution of live bearing in fishes. *Philosophical Transactions of the Royal Society, B: Biological Sciences, 357*, 259–267.

Grier, H. J., Fitzsimons, J. M., & Linton, J. R. (1978). Structure and ultrastructure of the testis and sperm formation in goodeid teleosts. *Journal of Morphology, 156*, 419–437.

Hara, M., Akagawa, I., & Kawahara, R. (2013). Comparative morphology of spermatozoa in the Gasterosteoidae. *Japanese Journal of Ichthyology, 60*, 1–13.

Hara, M., & Okiyama, M. (1998). An ultrastructural review of the spermatozoa of Japanese fishes. *Bulletin of the Ocean Research Institute, University of Tokyo, 33*, 1–138.

Harcourt, A. H., Purvis, A., & Liles, L. (1995). Sperm competition: Mating system, not breeding season, affects testes size of primates. *Functional Ecology, 9*, 468–476.

Hayakawa, Y., & Munehara, H. (1998). Fertilization environment of the non-copulating marine sculpin, *Hemilepidotus gigarti*. *Environmental Biology of Fishes, 52*, 181–186.

Heinen-Kay, J. L., & Langerhans, R. B. (2013). Predation-associated divergence of male genital morphology in a livebearing fish. *Journal of Evolutionary Biology, 26*, 2135–2146.

Heinicke, M. P., Naylor, G. J. P., & Hedges, S. B. (2009). Cartilaginous fishes (Chondrichthyes). In S. B. Hedges & S. Kumar (Eds.), *The tree of life* (pp. 320–327). Oxford University Press. http://php.scrip.tsu.edu/dept/evobio/hedgeslab/pubs/216.pdf

Hothorn, T., Bretz, F., Westfall, P., Heiberger, R. M., Schuetzenmeister, A., Scheibe, S., & Hothorn, M. T. (2016). *Package `multcomp`. Simultaneous inference in general parametric models*.

Humphries, S., Evans, J. P., & Simmons, L. W. (2008). Sperm competition: Linking form to function. *BMC Evolutionary Biology, 8*, 1–11.

Immler, S., Pitnick, S., Parker, G. A., Durrant, K. L., Lüpold, S., Calhim, S., & Birkhead, T. R. (2011). Resolving variation in the reproductive tradeoff between sperm size and number. *Proceedings of the National Academy of Sciences of the United States of America, 108*, 5325–5330.

Immler, S., Saint-Jalme, M., Lesobre, L., Sorci, G., Roman, Y., & Birkhead, T. R. (2007). The evolution of sperm morphometry in penguins. *Journal of Evolutionary Biology, 20*, 1008–1014.

Ishijima, S., Hara, M., & Okiyama, M. (1998). Comparative studies on spermatozoon motility of Japanese fishes. *Bulletin of the Ocean Research Institute, University of Tokyo, 33*, 139–152.

Ito, T., & Awata, S. (2019). Optimal methods to fix fish sperm for optical microscopic observation: Comparisons among different fixative solutions using sperms of copulatory and non-copulatory marine fishes. *Ichthyological Research, 66*, 307–315. https://doi.org/10.1007/s10228-018-0672-1

Ito, T., Kinoshi, I., Tahara, D., Goto, A., Tojima, S., Sideleva, V. G., Kuchinsky, A. B., & Awata, S. (2021). Fertilization modes drive the evolution of sperm traits in Baikal sculpins. *Journal of Zoology, 314*, 20–30.

Izumiyama, M., Awata, S., & Crow, K. (2020). Evaluating reproductive strategies and female bateman gradients in *Ditrema temminckii*: Is the number of fathers a good approximation for the number of mates? *Copeia, 108*, 532–537. https://doi.org/10.1643/CE-19-271.full

Jameson, B. G. M. (1987). *The ultrastructure and phylogeny of insect spermatozoa*. Cambridge University Press.
Yang, H., Hazlewood, L., Walter, R. B., & Tiersch, T. R. (2006). Effect of osmotic immobilization on refrigerated storage and cryopreservation of sperm from a viviparous fish, the green swordtail Xiphophorus helleri. *Cryobiology, 52*, 209–218.

Yao, Z., & Crim, L. W. (1995). Copulation, spawning and parental care in captive ocean pout. *Journal of Fish Biology, 47*, 171–173.

Yao, Z., Emerson, C. J., & Crim, L. W. (1995). Ultrastructure of the spermatozoa and eggs of the ocean pout (*Macrozoarces americanus* L.), an internally fertilizing marine fish. *Molecular Reproduction and Development, 42*, 58–64.

Zadmajid, V., Myers, J. N., Sørensen, S. R., & Butts, I. A. E. (2019). Ovarian fluid and its impacts on spermatozoa performance in fish: A review. *Theriogenology, 132*, 144–152. https://doi.org/10.1016/j.theriogenology.2019.03.021

Zeng, Y., Lou, S. L., Liao, W. B., & Jehle, R. (2014). Evolution of sperm morphology in anurans: Insights into the roles of mating system and spawning location. *BMC Evolutionary Biology, 14*, 104.

**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Ito, T., Morita, M., Okuno, S., Inaba, K., Shiba, K., Munehara, H., Koya, Y., Homma, M., & Awata, S. (2022). Fertilization modes and the evolution of sperm characteristics in marine fishes: Paired comparisons of externally and internally fertilizing species. *Ecology and Evolution, 12*, e9562. https://doi.org/10.1002/ece3.9562