Mating Season, Egg-Laying Season, and Internal Gametic Association in the Sympatrically Occurring Fluffy Sculpin (*Oligocottus snyderi*) and Rosy Sculpin (*O. rubellio*)

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Some marine sculpins (Psychrolutidae) exhibit an unusual reproductive mode called internal gametic association (IGA), in which sperm transfer between the sexes occurs during copulation, but fertilization is delayed until the eggs are released in seawater. IGA is suggested in many internally inseminating marine sculpins, but experimental evidence of IGA is limited to a few species. The Fluffy Sculpin (*Oligocottus snyderi*) and its sister species, the Rosy Sculpin (*O. rubellio*), occur in sympatry in intertidal zones along the central California coast. Although these species likely exhibit internal insemination, their reproductive strategy is not well understood. Here, we investigate reproductive mode, mating season, egg-laying season, and sperm morphology and activity in Fluffy and Rosy Sculpins near Pillar Point, California. Delayed embryonic development was observed for one clutch of eggs of the Rosy Sculpin after exposure to seawater, indicating IGA in this species. We were unable to demonstrate IGA by initiation of development in the Fluffy Sculpin because we were unable to collect females with ovulated oocytes. Nevertheless, we found that sperm morphology with elongated head and high motility in isotonic solution while immotile in seawater in both species represent characteristics associated with IGA. Seasonal changes in gonadosomatic index (GSI) of both sexes revealed asynchronous gonadal maturation between the sexes in the Fluffy Sculpin and suggest a similar pattern in the Rosy Sculpin; however, the latter was affected by small sample size. These patterns indicate that males copulate with females before egg maturation, and females store sperm for several months. Our study supports the generality of IGA across marine sculpins and provides an understanding of its relationship to asynchrony in GSI between the sexes. Further, while Fluffy and Rosy Sculpins are similar in body morphology, habitat, and reproductive mode, the slight difference in mating season (pre-mating isolation) and sperm head and flagellum length (post-mating isolation) may have contributed to divergence in sympatry with reduced probability of hybridization.

WHILE most oviparous fishes are broadcast spawners, in which eggs and sperm are released into the water column followed by external fertilization, some exhibit internal insemination with copulatory behavior. Fishes with internal insemination either exhibit true internal fertilization or an unusual reproductive mode called internal gametic association (IGA, Munehara et al., 1989). In species with IGA, sperm transfer occurs during copulation, where a spermatozoon associates with the oocyte micropyle in the ovary (Koya et al., 2002). However, fertilization is delayed until the eggs are released in seawater (Munehara et al., 1989, 1991; Petersen et al., 2005; Koya et al., 2015). Therefore, while IGA species exhibit copulation, they do not exhibit internal fertilization. IGA was first confirmed in the Elkhorn Sculpin (*Alcichthys alcicornis*, superfamily Cottoidea: Munehara et al., 1989), and this reproductive mode was later found in several species in the Cottoidea (e.g., Abe and Munehara, 2009 for review; Koya et al., 2015). Further, circumstantial evidence suggests that several species in other families may also exhibit an IGA-like reproductive mode. Histological studies of these other families indicate that ovaries contain spermatozoa, but neither developmental oocytes nor early cleavage stages are found in the ovary (e.g., Auchenipteridae: Burns et al., 2002; Parreira et al., 2009; Characidae: Burns et al., 1997). Akagawa et al. (2008) also suggested that the Japanese Tubesnout, *Aulichthys japonicus* (Aulorhynchidae, Gasterosteiformes), is

likely to exhibit IGA from behavioral observations and a previous report (Okiyama et al., 1993, but this statement was cited from a symposium and the content could not be confirmed). Although the actual timing of fertilization has not been studied in these species, they probably exhibit a reproductive pattern similar to that of IGA. Such IGA and IGA-like reproductive modes evolved convergently in multiple lineages and therefore represent a good model for an evolutionary strategy of copulation (Abe and Munehara, 2009).

The superfamily Cottoidea previously contained seven families (Nelson, 2006; Nelson et al., 2016). However, according to Smith and Busby (2014), based on phylogenetic analysis using both morphological and molecular data, the revised taxonomy of Cottoidea recognizes six families, one of which (Psychrolutidae) includes the former Bathylutichthyidae. In this revised taxonomy, nearly all former marine cottid genera (marine sculpins) and the former Psychrolutidae are included in Psychrolutidae, and the majority of freshwater cottid genera (i.e., freshwater sculpins) are assigned to the Cottidae. Although the grouping of the revised taxonomy is appropriate, considering that 190 species of the traditional marine Cottidae phylogenetically include 35 psychrolutids, it would be more appropriate to group them together as Cottidae, or to give them a new family name, or to make marine Cottidae and freshwater Cottidae as separate subfamilies of Cottidae. We suggest renaming Cottidae and Psychrolutidae to other family name but followed the

revised taxonomy of Smith and Busby (2014) here. Therefore, marine sculpins are treated as Psychrolutidae.

Of the six families in superfamily Cottoidea, three families (Agonidae, Cottidae, and Psychrolutidae) include both internal insemination and/or true external fertilization (Abe and Munehara, 2009). In the Cottidae, most species exhibit true external fertilization but two species of the genus *Comephorus* have acquired internal fertilization and viviparity (e.g., Jakubowski et al., 2003; Ito et al., 2021). Psychrolutidae (marine sculpins) and Agonidae exhibit both true external fertilization and internal insemination with copulation (Abe and Munehara, 2009). In Psychrolutidae and Agonidae, previous studies have shown that all of the internally inseminating species examined experimentally exhibit IGA (Koya et al., 1993, 2015; Abe and Munehara, 2005, 2009). Species with IGA share several characteristics with true internal fertilizers. For example, most IGA species have a well-developed genital papilla for mating (Evans and Meisner, 2009) and have sperm with an elongated head, in comparison with external fertilizing species that lack copulatory anatomy and exhibit round sperm-head morphology (Koya et al., 2011; Buser et al., 2017; Ito and Awata, 2019; Ito et al., 2022). While IGA is interesting in the evolution of reproductive strategies, it is currently unknown how widespread this strategy is within the Agonidae and Psychrolutidae. At present, 24 genera of agonids and psychrolutids are known to perform internal insemination spanning multiple independent clades (Agonidae: *Agonomalus*, *Blepsias*, *Brachyopsis*, *Hemitripterus*, *Ocella*, *Pallasina*, and *Podothecus*; Psychrolutidae: *Alcichthys*, *Artedius*, *Astrocottus*, *Bero*, *Chitonotus*, *Clinocottus*, *Enophrys*, *Furcina*, *Icelus*, *Oligocottus*, *Orthonopias*, *Pseudoblennius*, *Psychrolutes*, *Radulinopsis*, *Synchirus*, and *Vellitor*; see Abe and Munehara, 2009; Munehara, 2011; Koya et al., 2015; Momota and Munehara, 2017; Awata et al., 2019, 2022). However, only seven species have been experimentally demonstrated to exhibit IGA by observation of initiation of development via exposing the eggs to seawater (*Al. alcicornis*, Munehara et al., 1989; *Ar. harringtoni*, Petersen et al., 2005; *Bl. cirrhosus*, Munehara et al., 1991; *H. villosus*, Munehara et al., 1997; *Pa. barbata*, Momota and Munehara, 2017; *Po. sachi*, Munehara, 1997; *V. centropomus*, Koya et al., 2015).

Marine sculpins in the subfamily Oligocottinae (Psychrolutidae) are represented by 16 species including both external fertilizers and internal inseminators (Buser et al., 2017). They are all found in shallow waters of the west coast of North America and possess a suite of traits that enable them to exploit intertidal habitats (Ramon and Knope, 2008; Buser and López, 2015). Within the Oligocottinae, species of *Oligocottus* are dominant in shallow tidepools (Green, 1971; Miller and Lea, 1972; Nakamura, 1976). Historically, the Fluffy Sculpin (*Oligocottus snyderi*) was reported to exhibit internal fertilization in 1956 (Morris, 1956), and several studies have focused on the reproduction of the Fluffy Sculpin (Morris, 1956; Nakamura, 1976; Grossman and DeVlaming, 1984; Freeman et al., 1985). However, to our knowledge, neither IGA nor variation in gonadosomatic index (GSI), which is important to understand reproductive strategies, has been investigated. There are also no studies on reproduction/reproductive mode in the Rosy Sculpin (*O. rubellio*), which is the sister species of the Fluffy Sculpin and occurs in sympatry with the Fluffy Sculpin (Miller and Lea, 1972). In this study, we focused on reproductive strategies

of these two species, such as IGA and seasonal variation in gonad maturation (i.e., GSI) for both sexes. Evaluating reproductive strategies in a comparative context between these two sister species informs both the evolution of complex reproductive modes as well as mechanisms of reproductive isolation and speciation.

Seasonal variation in GSI can be used to infer mating season (i.e., when male gonads are ripe as indicated by max GSI) and egg-laying season (i.e., max GSI in females indicating the presence of mature oocytes) to reveal aspects of reproductive mode and strategy. In external fertilizers such as seasonal iteroparity, peak GSI is synchronized between males and females (e.g., Valdés et al., 2004; Martyniuk et al., 2009; Awata et al., 2010; Ochi et al., 2017; Kunishima et al., 2021; Samejima et al., 2021). Conversely, internally fertilizing viviparous fishes show offset peak GSI between the sexes, as females develop offspring after copulation (Yokogawa and Iguchi, 1992a; Izumiyama et al., 2020a, 2020b). Reproduction of female Fluffy Sculpins is characterized at one site (Dillon Beach, CA, USA) based on seasonal growth and oocyte maturation (Grossman and DeVlaming, 1984), indicating that egg laying occurs in winter and spring and is correlated with day length. However, seasonality of reproduction in male Fluffy and male and female Rosy Sculpins has not been characterized. Therefore, it is currently unknown whether mating season and egg-laying season are synchronized between the sexes, or if there is disparity in mating season between these sympatric sister species.

Here, we investigate the seasonal variation in male and female GSIs in Fluffy and Rosy Sculpins at Pillar Point, Half Moon Bay, California to estimate their mating and egg-laying seasons. Second, we verified species identity of Rosy and Fluffy Sculpins based on morphological characteristics and *CO1* and *Cytb* gene trees. Third, we experimentally investigated IGA in the two species to inform whether IGA is a common strategy across copulatory sculpins. Finally, we characterized sperm morphology and motility of both species to evaluate whether sperm morphology and kinematics are associated with the IGA strategy.

MATERIALS AND METHODS

Sampling.—Fluffy and Rosy Sculpins reach sexual maturity at approximately 40 mm standard length (SL, Grossman and DeVlaming, 1984; Freeman et al., 1985). Based on this, we collected 338 sexually mature sculpins of mixed species larger than about 40 mm SL during tractable tidal heights (–0.3 ft to +0.5 ft) in March 2018 and almost every month from October 2019 to May 2022 in the intertidal zone of rocky shores near Mavericks Beach at Pillar Point, Half Moon Bay, San Mateo County, California, USA (37°29′42.30″N, 122°29′53.03″W). One Smoothhead Sculpin (*Artedius lateralis*) and ten Wooly Sculpin (*Clinocottus analis*) were also collected at the same site for genetic analyses.

Species identification.—The external morphology of Fluffy and Rosy Sculpins is very similar, especially in females (Nakamura, 1976), making species field identification challenging. Therefore, it was necessary to confirm species identity by DNA sequencing for the specimens with less certainty. Genomic DNA was extracted from fin clips taken from each individual using Qiagen DNEasy blood and tissue kit (Qiagen Inc., Valencia, CA), and the *Cytb* and *CO1* loci were

Table 1. Primers used in the analysis of the cottid mitochondrial genome.

Primer	mtDNA region	Sequence 5′–3′	Reference
H15915/L14724	*Cytb*	H15915—CAACGATCTCCGGTTT L14724—GTGACTTGAAAAACCA	Schmidt and Gold (1993)
FISH_F1/FISH_R1	*CO1*	FISH_F1—TCAACCAACCACAAAGACATTGGCAC FISH_R1—TAGACTTCTGGGTGGCCAAAGAATCA	Ward et al. (2005)

sequenced to confirm species identity in a phylogenetic context. Gene trees were constructed using 561 bp of *CO1* (*n* = 209 fish of mixed species, including one Smoothhead and ten Wooly Sculpins) and 1,051 bp of *Cytb* (*n* = 38 fish). Primer sequences (Table 1) were adopted from Ward et al. (2005) or Schmidt and Gold (1993). *CO1* amplifications were accomplished following Buser and López (2015). PCR products were purified using Qiagen PCR purification kit and sequencing reactions were performed by ElimBio Pharmaceuticals (Hayward, CA). PCR amplification and sequencing of the *Cytb* locus were accomplished following Awata et al. (2019, 2022).

Gene tree topologies were constructed using MEGA version X (Stecher et al., 2020), with maximum likelihood with a GTR+G+I model of molecular evolution and 1,000 bootstrap replicates. For species identification, 11 *CO1* sequences from ten species and 19 *Cytb* sequences from ten species were obtained from NCBI (Tables S1, S2; see Data Accessibility; Hastings and Burton, 2008; Buser and López, 2015; Turanov and Kartavtsev, 2021; Awata et al., 2022). For both trees, *Scorpaenichthys marmoratus* was set as the outgroup.

Embryonic development and evaluation of IGA.—We used the onset of development to indicate IGA in Rosy Sculpin and characterized the first 21 days of embryonic development, following the methods of Munehara et al. (1989, 1991), Petersen et al. (2005), and Koya et al. (2015). After euthanization of a gravid female of Rosy Sculpin (*n* = 1) with an overdose of MS-222 (200 mg/L) followed by cervical transection, eggs were stripped from the female by pushing on the abdomen with no contamination from seawater, blood, or urine. The egg clutch was split into two portions, and each was placed into experimental and control petri dishes with covers. Seawater was added to the experimental dish but not to the control dish. The control dish contained eggs with ovarian fluid and moistened Kimwipes. The dishes were floated on the top of seawater to regulate the temperature (13–15°C) and humidity. Early developmental progress was checked daily before egg hatching. We also attempted to assess IGA and egg development of five females of Fluffy Sculpin but did not find any females with ovulated oocytes in the ovaries (see Results).

Characterization of sperm motility and morphology.—To evaluate the association between sperm characteristics and IGA, sperm motility and morphology were evaluated using males of Fluffy (*n* = 8) and Rosy (*n* = 3) Sculpins. Testes were dissected from the euthanized male, semen was collected by cutting the posterior region of the testis, and the semen was diluted in seawater or isotonic solution (150 mM NaCl, 10 mM HEPES, pH 8.0, following Ito and Awata [2019] and Koya et al. [1993] in a 1:30 ratio to mimic ovarian fluid) on glass slides coated with 1% bovine serum albumin. Sperm motility was recorded using a phase-contrast microscope (DSM-III-104, Daiko Science, Japan), equipped with a digital CCD camera (HD 212, AmScope, USA). Sperm swimming speed (measured as curvilinear velocity) was calculated using cell motility analysis software (BohBoh version 4.51J, BohBohSoft, Japan). A small amount of semen was preserved in a fixative of 2.5% glutaraldehyde, 0.45 M glucose, and 60 mM HEPES, following Ito and Awata (2019) for characterization of sperm morphology. We photographed sperm using a differential interference microscope (BX53, Olympus, Japan), equipped with a digital color CCD camera (DP73, Olympus, Japan) and CellSens Standard software ver. 1.9 (Olympus, Japan). We measured the morphology of the sperm components in the images using ImageJ ver. 1.50i (Schneider et al., 2012). Fine structure of the sperm of Fluffy Sculpin (*n* = 1) was also photographed using a scanning transmission electron microscope (Zeiss Ultra 55, GEMINI technology, England).

Calculation of gonadosomatic index (GSI).—Total body mass, gut mass, and gonad mass (to 0.001 g) were recorded for each euthanized individual to examine seasonal variation in gonadosomatic index (GSI, $Mass_{gonad}/[Mass_{body}-Mass_{gut}] \times 100$) of Fluffy and Rosy Sculpins. GSI was calculated as gonad mass divided by total body mass minus gut mass to remove variation associated with feeding. Male and female GSI were plotted for each month to infer mating and egg-laying seasonality, regardless of sampling year. The monthly average water temperature at the study site was obtained from the Sea Temperature website (https://seatemperature.net/current/united-states/half-moon-bay-sea-temperature) to evaluate effects of temperature on mating- or egg-laying seasons.

Statistical analyses.—For the sperm morphological characteristics, we measured total sperm length (µm), flagellum length (µm), head length and width (µm), and midpiece length and width (µm); we also calculated head length/head width, head length/total sperm length, head length/flagellum length, and midpiece length/midpiece width. We compared these ten variables and sperm swimming speed between Fluffy and Rosy Sculpins. In these comparisons, linear mixed models (LMMs) were fitted considering species as fixed effects and individual ID as a random effect, given the measurement of each characteristic per male from multiple sperms. Likelihood ratio tests were performed to determine the statistical significance ($P < 0.05$) of the fixed effects. Statistical analyses were conducted using R software (R Core Team, 2016).

RESULTS

Species identification.—As has been described in Morris (1956), male Fluffy and Rosy Sculpins exhibit an anal fin modification, where the first two anal soft rays are distinct and elongated for optimization of copulation, with slight morphological variation between species (Fig. S1; see Data Accessibility). Buser et al. (2017) identified morphological variation between the species, such as body depth, eye size,
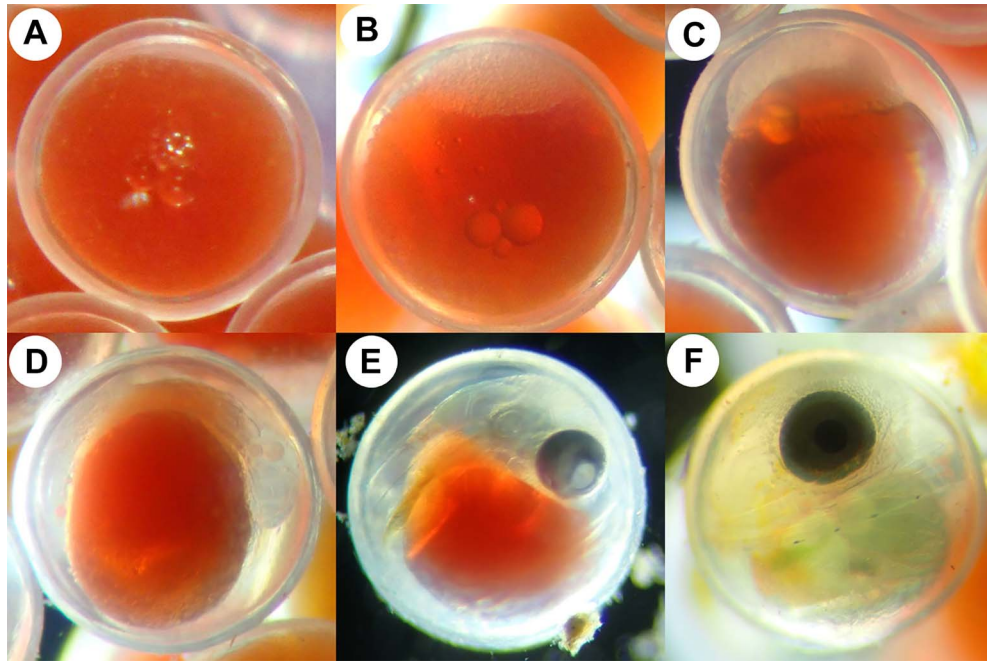
Fig. 1. Embryonic development of the Rosy Sculpin (*Oligocottus rubellio*). (A) Eggs were immersed in seawater (Fertilization). (B) Blastula stage (24 hours post fertilization, hpf). (C) Early gastrula stage (48 hpf). (D) Phylotypic stage (6 days post fertilization, dpf). Optic and otic vesicles were visible. (E) Embryonic stage (11 dpf). Eye pigmentation and otoliths were clearly visible. (F) Embryonic stage just before hatching (21 dpf). Lateral line pigmentation was visible. Water temperature was maintained at 13–15°C.

and shape, but these morphological characteristics are difficult to discern in the field, especially in females. Therefore, we verified species identification by using the reconstructed phylogeny of the specimens. Gene trees of *CO1* and *Cytb* were diagnostic for the two species (Figs. S2, S3; see Data Accessibility). In the *CO1* phylogenetic trees (Fig. S2; see Data Accessibility), 138 samples were assigned to the Fluffy Sculpin, including two samples (1.5%) being misidentified as the Rosy Sculpin during field morphological identification. Such a misidentification rate was higher for the Rosy Sculpin (Fig. S2; see Data Accessibility); of 60 genetically assigned to the Rosy Sculpin, 21 (35.0%) were morphologically assigned as the Fluffy Sculpin in the field. In the *Cytb* phylogenetic trees (Fig. S3; see Data Accessibility), 21 and 17 samples were assigned as the Fluffy and Rosy Sculpin, respectively. We also found that the *CO1* sequence of the Fluffy Sculpin registered in GenBank (KP827314: Buser and López, 2015) was misidentified as the Woolly Sculpin. The *Cytb* sequences of Rosy Sculpins were deposited in GenBank (*n* = 5 fish; Table S2; see Data Accessibility). In total, 90 males and 150 females of Fluffy Sculpin and 36 males and 62 females of Rosy Sculpin were verified from the morphological and genetic analyses.

IGA and embryonic development.—Mean ± SD of ovulated oocyte diameter was 1.33±1.01 mm for Rosy Sculpin (*n* = 11 eggs from one female). After exposing a portion of an egg mass to seawater (experimental dish), the two-cell stage of the embryo was observed within 10 h (Fig. 1A). The blastula stage and early gastrula stage were observed after 24 and 48 h, respectively (Fig. 1B, C). Optic vesicles were developed by six days post fertilization (dpf), and eye pigmentation and otoliths were observed at 11 dpf (Fig. 1D, E). Well-developed embryos with lateral-line pigmentation hatched

at approximately 22 dpf (Fig. 1F). Development within the clutch was uniform, suggesting a single fertilization event for the entire clutch (with no inference on number of sperm donors). No developmental eggs were observed in the control dish even after 24 h. We were unable to observe embryonic development in the Fluffy Sculpin in five different experimental trials with five females due to the lack of ovulated oocytes in the females at the time of collection for the IGA experiment. The ovaries of these five individuals contained eggs of several size classes, but none were free from the ovarian matrix. Attempts were made to expose these eggs to seawater, but no development was observed in any of the five clutches.

Sperm characteristics.—Sperm morphology in both Fluffy and Rosy Sculpins was similar in appearance (Fig. 2A, B). The morphology of the head was elongated, not spherical, and had a 'butter knife' shape. In the Fluffy Sculpin, fine structure imaging revealed that the flagellum was covered by the midpiece and recessed into the head (Fig. 2C). Although there was a high degree of similarity in sperm morphology between species, there were significant differences in five morphometric components (Table 2). Total sperm length and flagellum length of Rosy Sculpin were significantly longer than those of Fluffy Sculpin. Sperm head length and width in Rosy Sculpins were also significantly longer than those in Fluffy Sculpins, but the head shape (head length/head width) did not differ between the species. Midpiece length, width, and their ratio were not significantly different between the species. The ratio of head length to total sperm and to flagellum length also differed slightly between the species. In both Fluffy and Rosy Sculpins, sperm were motile in isotonic solution but completely immotile in seawater. Sperm swimming speed was not significantly different between the two species (Table 2).
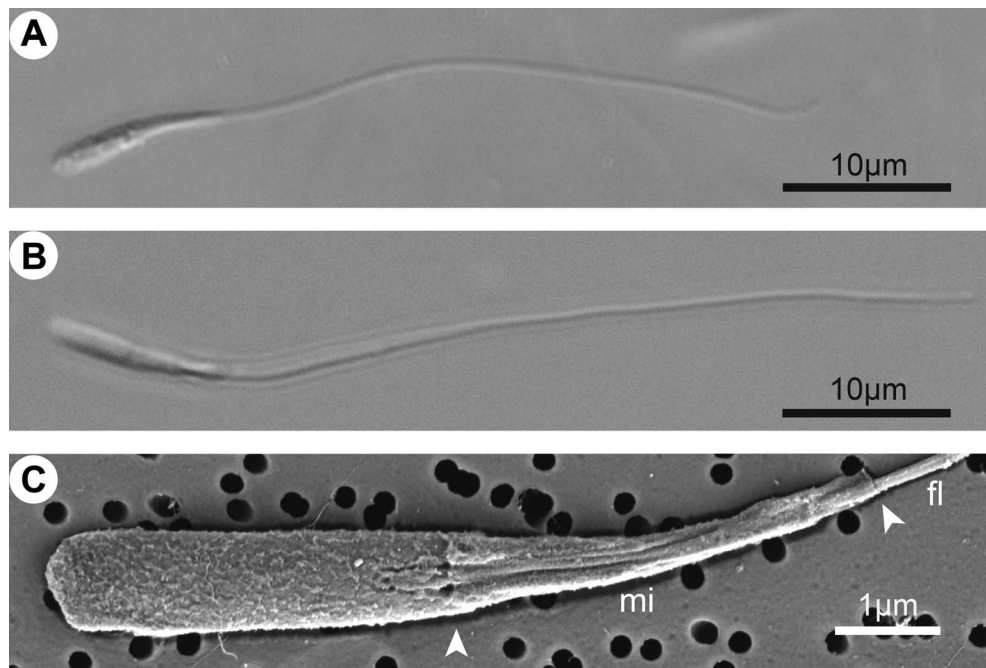
Fig. 2. Sperm of (A) the Fluffy Sculpin (*Oligocottus snyderi*) and (B) the Rosy Sculpin (*O. rubellio*) observed under a differential interference microscope. (C) The fine structure of sperm head in the Fluffy Sculpin observed under a scanning transmission electron microscope. fl: flagellum, mi: midpiece. The left and right white arrows indicate the boundary between head and anterior end of the midpiece and between anterior and posterior ends of the midpiece, respectively.

Seasonal changes in GSI.—The monthly average sample size used for the GSI analyses was 8 males and 13 females in Fluffy Sculpins. There was seasonal variation in male and female GSIs of Fluffy Sculpins (Fig. 3). We define mating season by elevated GSI that was > c.a. 1.5% of reproductively active males (> 40 mm SL). According to this criterion, we estimated that the mating season for Fluffy Sculpin is protracted, spanning a six-month period from September to February (Fig. 3A). GSI values for males shows that peak mating occurs from October to December.

As expected from the reproductive mode in the Fluffy Sculpins, asynchronous gonadal maturation between the sexes was observed. GSI values for female Fluffy Sculpin were elevated from November to May, with peak egg-laying season occurring from December to March (with average

GSI ranging from 8.4 to 9.1%, Fig. 3B). Unfortunately, the five female Fluffy Sculpins we examined for IGA development trials did not include any individuals with ovulated oocytes. Females exhibited lower GSI values from June to October. Note that females were much less abundant in the intertidal site during this time, suggesting that they may have died after laying eggs, or migrated away from the intertidal, suggesting the intertidal is used as a nursery habitat for these species.

Although the sample size of Rosy Sculpins was too small (3 males and 5 females on monthly average) to make strong inferences due to their low abundance at our study site, seasonal variation in GSI values were also observed for both male and female Rosy Sculpins. The GSI values of males increased from November to May (i.e., mating season, Fig. 3C).

Table 2. Sperm morphological characteristics and swimming speed in Fluffy Sculpin (*Oligocottus snyderi*) and Rosy Sculpin (*O. rubellio*). All values represent mean ± SD. Parentheses show the number of individuals (left) and sperm cells (right) used for the analyses. Linear mixed models were performed, setting individual ID as a random effect.

| Sperm characteristics	*O. snyderi*	*O. rubellio*	Statistics χ^2	P
Total sperm length (µm)	44.36±1.29 (9, 70)	52.99±1.19 (3, 23)	30.00	<0.0001
Head length (µm)	5.2±0.32 (8, 53)	5.6±0.3 (3, 23)	4.63	0.031
Head width (µm)	1.36±0.17 (7, 51)	1.81±0.36 (3, 23)	6.71	0.009
Midpiece length (µm)	4.42±0.49 (8, 52)	4.55±0.77 (3, 23)	0.49	0.49
Midpiece width (µm)	0.99±0.14 (7, 50)	1.08±0.06 (3, 23)	2.55	0.11
Flagellum length (µm)	39.57±1.67 (8, 52)	47.39±1.47 (3, 23)	21.34	<0.0001
Head length/head width	3.91±0.67 (7, 51)	3.35±0.37 (3, 23)	1.93	0.17
Midpiece length/midpiece width	4.76±0.54 (7, 50)	4.25±0.96 (3, 23)	0.79	0.38
Head length/total sperm length	0.12±0.01 (8, 52)	0.11±0.01 (3, 23)	4.06	0.044
Head length/flagellum length	0.13±0.01 (8, 52)	0.12±0.01 (3, 23)	3.79	0.052
Sperm swimming speed (µm/s)	75.61±19.41 (6, 118)	91.81±2.23 (2, 25)	1.48	0.22
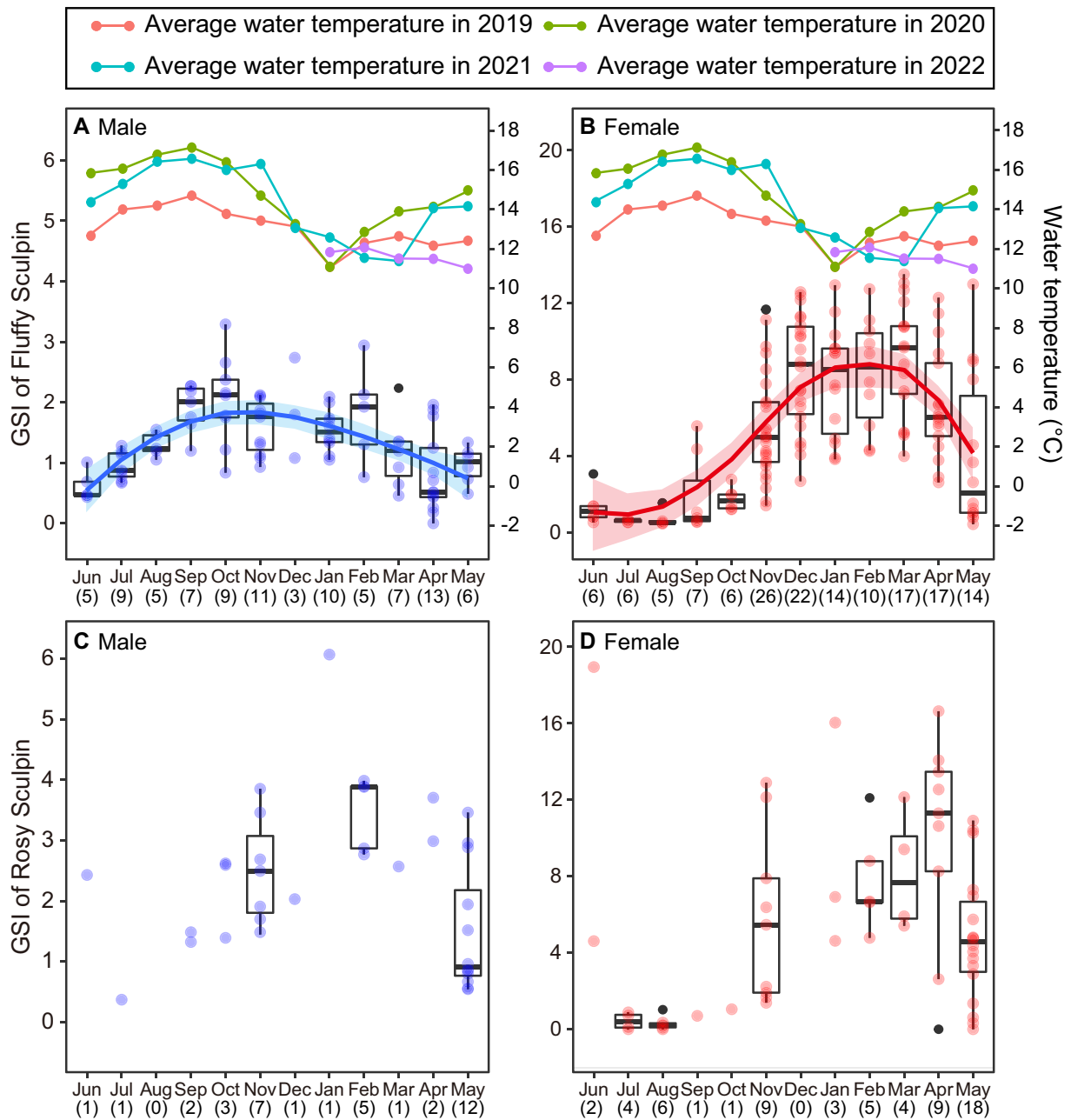
Fig. 3. Seasonal changes of gonadosomatic index (GSI) in males and females of the Fluffy Sculpin (*Oligocottus snyderi*) and the Rosy Sculpin (*O. rubellio*) and monthly average water temperature at study site in Half Moon Bay, San Mateo County, CA, USA. (A) Male (*n* = 90) and (B) female (*n* = 150) Fluffy Sculpin. (C) Male (*n* = 36) and (D) female (*n* = 62) Rosy Sculpin. Each dot represents an individual. In boxplots, boxes show median, interquartile range, and whiskers show the lowest/highest value within 1.5 times the interquartile range, with black dots of outlier. Boxplots were illustrated only if the number of individuals collected per month was four or more. Solid lines indicate the loess regressions, and shadings indicate 95% confidence intervals. The number below the month denotes the number of individuals.

Egg-laying season of Rosy Sculpins, indicated by the increase in female GSI, occurred from January to June (Fig. 3D). The water temperature ranged from 11 to 16°C in 2019–2022, and female GSI of both species had an inverse relationship with water temperature (Fig. 3B, D).

DISCUSSION

Embryonic development was observed in eggs of the Rosy Sculpin exposed to seawater but not for those only in the ovarian fluid, indicating that the Rosy Sculpins exhibit the IGA reproductive strategy. We also describe, for the first

time, aspects of embryonic development in Rosy Sculpin, which hatched at approximately 23 days after contact with seawater. Unfortunately, we failed to obtain mature female Fluffy Sculpins with ovulated oocytes for the IGA experiments. However, we also did not find any fertilized eggs or eggs of early cleavage stages in the ovaries of the Fluffy Sculpin. The ovaries contained spermatozoa, indicating that copulation had taken place, but fertilization had not occurred. Further, the sperm morphology of Fluffy Sculpins is characterized as having an elongated head and motility in isotonic solution but immotile in seawater, similar to the sperm characteristics of Rosy Sculpins and consistent with

characteristics associated with IGA species (Abe and Munehara, 2009; Koya et al., 2011; Ito and Awata, 2019; Ito et al., 2022). Therefore, although Morris (1956) has noted that the Fluffy Sculpins are internal fertilizers, we infer that their reproductive mode is IGA, as we observed in Rosy Sculpins.

Previous studies have shown that seven internally inseminating cottoid species have an IGA reproductive mode (Munehara, 1997; Munehara et al., 1989, 1991, 1997; Petersen et al., 2005; Koya et al., 2015; Momota and Munehara, 2017) and have suggested that other copulatory species are also likely to exhibit IGA (Abe and Munehara, 2009; Koya et al., 2015; Awata et al., 2019, 2022). The present results support this assumption and indicate that IGA evolved multiple times independently in multiple clades of the Cottoidea (Agonidae and Psychrolutidae). In addition, although the majority are external fertilization, IGA or IGA-like fertilization modes are recognized in a few species in perciforms (Cottoidea) as well as in characiforms (at least 27 species in Glandulocaudinae and Stevardiinae of Characidae: Burns et al., 1995, 1997), gasterosteiforms (one species in Aulorhynchidae: Akagawa et al., 2008; Okiyama et al., 1993), and siluriforms (at least five species in Auchenipteridae: Meisner et al., 2000; Burns et al., 2002; Parreira et al., 2009). The presence of IGA in a wide range of taxa suggests that IGA may have evolved independently across fishes of different families in multiple orders. In terms of life history strategies, species with IGA share several characteristics with both oviparous and viviparous fishes. First, oviparous fishes invest more in egg number than egg size (Sargent et al., 1987), resulting in higher larval mortality compared to viviparous fishes (Smith and Fretwell, 1974; Roff, 1993). This theory is also predicted to apply to species with IGA because the females spawn egg masses into seawater and eggs are exposed to various extraneous factors. Second, females of IGA species do not need to care for fertilized eggs and larvae in the ovary. This characteristic is also similar to oviparous fishes by spawning eggs in seawater and by reproductive costs in the ovary that are predicted to be lower than those in viviparous fishes (Abe and Munehara, 2009), although the total reproductive cost, including parental care, is unknown. Third, females can lay eggs at the appropriate timing of spawning and do not need to synchronize their spawning timing with males, especially for females that produce multiple clutches in a reproductive season (Abe and Munehara, 2009). In terms of asynchrony between copulation and spawning, this character is similar to asynchrony between copulation and birth in viviparous fishes. Evolution of IGA may allow females to have decoupled nest site selection from mating, giving them time to decide when and where to lay eggs, without the cost of rearing larvae and juveniles in the ovary. Although it is unclear what factors separate the evolutionary pathways from external fertilization to IGA or true internal fertilization in fishes, accumulating evidence of IGA in different clades of fishes could contribute to our understanding of the underlying mechanisms and potential implications of this reproductive strategy.

Aspects of sperm morphology, including head length, ratio of total length or flagellum length to head, and motility, were similar between Rosy and Fluffy Sculpins; both species had sperm with an elongated head and were motile in the isotonic solution but not in seawater. These characteristics contrast with those in external fertilizing species, which have round-head sperm and sperm that are motile in their fertilization environment such as seawater (Koya et al., 1993, 2011; Petersen et al., 2005; Abe and Munehara, 2007; Ito and Awata, 2019; Ito et al., 2022) and fresh water (Ito et al., 2021). Sperm with an elongated and slender head are characteristic of IGA species and is thought to be advantageous for swimming in a viscous environment such as ovarian fluid (Javonillo et al., 2009). Interestingly, Humphries et al. (2008) proposed that sperm with a longer head (ellipsoidal shape) exhibit increased the drag force, resulting in decreased sperm velocity. Revealing the detailed structure, not just the simple head-to-flagellum ratio, is important for clarifying the physical function of sperm; the thickness might also be associated with sperm swimming speed and adaptation to a viscous environment.

Mating and spawning periods in Fluffy Sculpins were not synchronized, as shown by divergence in peak GSI between males (September to December) and females (December to March). These results suggest that females store sperm for over three months before laying their first clutch of the season. In the absence of verified monogamy, it is reasonable to assume that females mate with multiple males resulting in clutch polyandry as the null model. Morris (1956) noted that, in captivity, copulation of the Fluffy Sculpin is frequently observed. Although polyandry in Fluffy Sculpin remains untested, females store sperm (from one or more males), and it is currently unknown when exactly fertilization takes place. Every female examined had oocytes at multiple stages of development at any one time (Grossman and DeVlaming, 1984), indicating that they lay multiple egg clutches per season. Therefore, it is not surprising that the mating season is protracted in both species. Asynchrony in peak GSI is observed in several sculpin species with IGA (Shinomiya, 1985; Abe and Munehara, 2005, 2007). Fluffy and Rosy Sculpins are sister taxa (Ramon and Knope, 2008; Buser and López, 2015; Buser et al., 2017) and overlap in geographic range and habitat, occupying the same pools in the intertidal (this study). Although our sample size for Rosy Sculpin was too small to reliably infer mating season, the possibility of divergence in mating season suggests a mechanism for reproductive isolation and divergence in sympatry.

In marine sculpins (Psychrolutidae), copulatory IGA species are classified into three egg-care patterns; paternal care, maternal care, and no care with egg masses deposited in invertebrates (Abe and Munehara, 2009; Awata et al., 2019, 2022). Such egg-care patterns may be related to synchronization of mating season and egg-laying season (Munehara, 2011). For example, the timing of copulation and spawning are close together in IGA species with paternal care (*Alcichthys alcicornis*, Munehara, 1988; *Artedius harringtoni*, Ragland and Fischer, 1987). Indeed, *Al. alcicornis* is likely to exhibit synchrony in peak GSI between the sexes (Koya et al., 1994; Munehara and Murahana, 2010). Alternatively, IGA species with maternal care (e.g., *Radulinopsis taranetzi*: Abe and Munehara, 2005) and those with no care (five species of *Pseudoblennius*, two species of *Furcina*, and one species of *Vellitor*: Koya et al., 2015; Awata et al., 2019; *Orthonopias triacis*: Awata et al., 2022) exhibit delayed timing of spawning from timing of mating. Males of *R. taranetzi* copulate with females that do not yet have mature oocytes about three months before the egg-laying season (Abe and Munehara, 2005, 2007). Females of *Vellitor centropomus* also

copulate while still immature (Koya et al., 2015), suggesting that sperm can be stored for several months. Viable sperm are retained for two weeks in *O. triacis*, demonstrating sperm storage capacity (Bolin, 1941). Although egg-care patterns in the Fluffy and Rosy Sculpins are unknown in the field (Awata et al., 2022), the asynchrony in peak GSI between the sexes implies that these species do not exhibit paternal-care behaviors. In fact, none of the males collected in this study had protected eggs. Female egg care is known only in species that inhabit sandy and gravel areas (Abe and Munehara, 2005, 2007). Many parts of the intertidal area are covered in pebble and are not unlike gravel. Therefore, we cannot rule out the possibility of maternal care, but as with the males, none of the females we collected were protecting their eggs.

Asynchrony in mating season and egg-laying season in marine sculpins is also suggested to be related to sperm competition (Munehara, 2011), which is the competitive process for fertilization between sperm from different males (Parker, 1970). Males can have a capacity of copulating earlier than when eggs are mature, thereby reducing the chance of fertilization by other males. Indeed, females of *R. taranetzi* mate with multiple partners, resulting in intense sperm competition in the ovary (Abe and Munehara, 2005, 2007). Although little information is available on parental egg care and variation in GSI for both males and females in marine sculpins, the asynchrony of the GSI may be the result of male–male competition because males that mate earlier may be able to fertilize more eggs or the first mature oocyte of multiple clutches (Abe and Munehara, 2009; Munehara et al., 1994; Munehara, 2011).

Asynchrony in peak GSI is also observed in internally inseminating catfishes (Freitas et al., 2011) and characid fishes (Azevedo et al., 2000) and internally fertilizing surfperches (Izumiyama et al., 2020b) and rockfishes (Yokogawa and Iguchi, 1992a, 1992b). While GSI is protracted and there is some overlap between the sexes in IGA species including Fluffy and Rosy Sculpins, GSIs between the sexes are less overlapped in true internal fertilizers (i.e., viviparous fishes). The protracted seasonality and disparity in GSI peaks between the sexes may be driven by the number of clutches laid per season. Viviparous fishes with seasonal iteroparity exhibit a single brood per year. Surfperch females copulate with multiple males, but fertilization and early embryonic development are initiated after mating season ends (Izumiyama et al., 2020a, 2020b); thus, asynchrony is obvious. Different from the surfperches, female Fluffy and Rosy Sculpins exhibit multiple size classes of oocytes within the ovary between October and May (Grossman and De-Vlaming, 1984), indicating multiple clutches are laid per season. Therefore, since males of the Fluffy and Rosy Sculpins have multiple chances to fertilize eggs due to the multiple spawnings by an individual female, the GSI peaks of both sexes would be prolonged and have slight overlap with each other.

In summary, we demonstrate seasonal variation in GSI between males and females of Fluffy and Rosy Sculpins. We also demonstrated IGA in Rosy Sculpin directly and inferred IGA in Fluffy Sculpin indirectly based on sperm morphology, asynchrony of GSI between the sexes, and lack of embryonic development in the ovary. This reproductive strategy of IGA together with protracted and asynchronous mating season could be associated with male–male competition, sperm storage, multiple spawning, and optimal nest site selection by females. Furthermore, we propose that the slight difference in mating season (pre-mating isolation) and sperm characteristics (post-mating isolation) may prevent hybridization between the two species, while they are sympatric and share similarities in morphology and reproductive mode. Our study supports the generality of IGA across marine sculpins and demonstrates the utility of these patterns for diagnosing the IGA reproductive strategy.

DATA ACCESSIBILITY

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