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The first record of egg masses in tunicates deposited by the snubnose sculpin, *Orthonopias triacis*, from the Northeastern Pacific: evidence for convergent evolution of an unusual reproductive strategy

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ABSTRACT

In this study, the authors report the first record of egg masses deposited in solitary tunicates by the snubnose sculpin, *Orthonopias triacis*, from the Northeastern Pacific. Four egg masses were discovered in the tunicate *Ascidia ceratodes* that were genetically determined to be *O. triacis*. Female *O. triacis* had long ovipositors that allow deposition of their eggs inside the atrium of the tunicates. A comparison of host-tunicate size with ovipositor length of sculpins from the Northwestern Pacific, including the genera *Furcina* and *Pseudoblennius*, revealed that *O. triacis* had shorter ovipositors and spawned in the atrium of smaller species of tunicates. Ancestral state reconstruction of egg deposition in solitary tunicates using 1.86Mbp RNAseq data of 20 sculpin species from Northeastern and Northwestern Pacific revealed that this unusual spawning behaviour may have evolved convergently in different species occurring in the Northeastern vs. the Northwestern Pacific.

KEYWORDS

California, Monterey, ostracophil, ovipositor, sculpin, tunicate

1 | **INTRODUCTION**

Some fishes deposit egg clutches in live invertebrates, and these species are known as ‘ostracophils’ (Balon, 1975; Leung, 2014). Ostracophils occur in both freshwater and marine habitats and are good models for understanding the co-evolution of host-parasite interactions in this context (reviewed in Karplus, 2014; Leung, 2014). Nonetheless, few studies have focused on the evolutionary adaptations in ostracophilous fishes. Most information on morphological and behavioural adaptations for ovipositing in their host has been accumulated for the bitterling–mussel system inhabiting freshwater systems (*e.g.,* Kitamura, 2007; Smith *et al.*, 2004), where female bitterlings (Cyprinidae: Acheilognathinae) deposit eggs in the gill chambers of freshwater mussels (Unionidae and Margaritiferidae). In this system, female bitterlings have extremely long ovipositors as an adaptation to oviposition (Kitamura, 2007; Kitamura *et al.*, 2012; Smith *et al.*, 2004). Among marine fishes, host invertebrates used by ostracophilous fishes are diverse. Blennies, marine sculpins, sea ravens, poachers and snailfishes oviposit in the spongocoels of sponges (Busby *et al.*, 2012; Chernova, 2014; Karplus, 2014; Momota & Munehara, 2017) and in the branchial chambers of crabs (*e.g.,* Gardner *et al.*, 2016; Hunter, 1969; Matsuzaki *et al.*, 2020; Poltev & Mukhametov, 2009), whereas the Japanese tubesnout *Aulichthys japonicus* Brevoort, 1862 (Akagawa *et al.*, 2008) and marine sculpins (Awata *et al.*, 2019; Goto & Oba, 2019; Nishida *et al.*, 2008) oviposit in the atria of tunicates. As has been found in freshwater bitterlings, ostracophilous snailfishes and sculpins have elongated ovipositors (Awata *et al.*, 2019; Hunter, 1969).

The marine sculpins (Psychrolutidae, 64 genera, 214 species) and sea ravens and poachers (Agonidae, 25 genera 59 species), that belong to the Infraorder Cottales (Betancur-R *et al.*, 2017; Nelson *et al.,* 2016; Smith & Busby, 2014), represent a diverse group exhibiting morphological variation associated with specific reproductive strategies (Abe & Munehara, 2009; Buser *et al.*, 2017; Knope, 2013; Koya *et al.*, 2011). They represent an ideal system to study morphological and behavioural adaptations driven by the interactions between parasites and hosts because several sculpins, sea ravens, and poachers are known to parasitize live invertebrates for egg deposition (Abe & Munehara, 2009; Busby *et al.*, 2012; Karplus, 2014; Momota & Munehara, 2017), and a recent study has shown that ostracophilous marine sculpins exhibit interspecific variation in host selection and female reproductive traits such as ovipositor length (Awata *et al.*, 2019). There are three patterns of host use by ovipositing sculpins including species that primarily use colonial tunicates, those that exclusively use solitary tunicates and those that use exclusively sponges (Awata *et al.*, 2019), showing host specificity. Furthermore, species that occur in sympatry use different host species (*e.g.,* tunicates or sponges) with different host sizes. The authors of this study proposed that ovipositor length may have co-evolved to accommodate morphology of specific host invertebrates (Awata *et al.*, 2019). Indeed, the ovipositor of species using solitary tunicates as hosts (four species of the genus *Pseudoblennius* and two species of the genus *Furcina*) is much longer than species using colonial tunicates and/or sponges [*Pseudoblennius marmoratus* (Döderlein, 1884) and *Vellitor centropomus* (Richardson, 1850): Awata *et al.*, 2019]. To the best of the authors' knowledge, however, only two studies have addressed this hypothesis directly (bitterlings: Kitamura *et al.*, 2012; marine sculpins: Awata *et al.*, 2019).

Among marine sculpins, sea ravens and poachers, many species, especially those inhabiting shallow waters, are endemic to the Northeastern or Northwestern Pacific (Kells *et al.*, 2016; Knope, 2013; Nakabo & Kai, 2013). These fishes exhibit a diversity of reproductive modes, including external mixing of gametes in non-copulating species and internal gametic association (IGA: sensu Munehara *et al.*, 1989) which occurs in copulating species with a characteristic of male enlarged genital papilla, where sperm are transferred from a male into the ovary of a female, but egg fertilization is delayed until the eggs are extruded into seawater.

Previous studies have shown that both copulating and non-copulating species are found in the Northeastern or Northwestern Pacific (Abe & Munehara, 2009; Buser *et al.*, 2017; Koya *et al.*, 2011), indicating convergent evolution of IGA on both sides of the Pacific (Abe & Munehara, 2009; Koya *et al.,* 2011; Yabe, 1985). IGA is observed in all known ostracophilous sculpins, sea ravens, and poachers (Abe & Munehara, 2009; Awata *et al.*, 2019; Momota & Munehara, 2017). If ostracophily are found in different sculpin species that are endemic to the Northeastern and Northwestern Pacific, ostracophily may have arisen multiple times independently from non-ostracophilous ancestors. Although some ostracophilous sculpins and sea ravens using sponges as hosts have been described in both the Northwestern Pacific [*Blepsias cirrhosis* (Pallas, 1814), but occurring in both the Northwestern and Northeastern Pacific: Munehara, 1991; *P. marmoratus* and *V. centropomus*: Awata *et al.,* 2019] and the Northeastern Pacific [*Hemitripterus bolini* (Myers, 1934): Busby *et al.*, 2012], marine sculpins that oviposit in solitary tunicates had only been found in the Northwestern Pacific [*Pseudoblennius* sp. "Kirin-anahaze," *Pseudoblennius cottoides* (Richardson, 1848), *Pseudoblennius percoides* Gunther, 1861, *Pseudoblennius zonostigma* Jordan et Starks, 1904, *Furcina ishikawae* Jordan et Starks, 1904, and *Furcina osimae* Jordan et Starks, 1904: Nishida *et al.*, 2008; Awata *et al.*, 2019; Goto & Oba, 2019]. Nonetheless, the authors found egg masses in the atrium of solitary tunicates in Carmel Bay, CA, USA in 2009, and that female snubnose sculpin *Orthonopias triacis* Starks & Mann, 1911 had long ovipositors, as noted by Bolin 80 years ago (Bolin, 1941). Furthermore, snubnose sculpins are a copulating species (Bolin, 1941; Koya *et al.*, 2011) and the authors of this study confirmed that they exhibit IGA (H. Munehara and Y. Koya, unpub. data), following the methods of Munehara (1991) and Koya *et al.* (2015). Nonetheless, the authors were unable to identify the parental species of those eggs found in tunicates using molecular markers. Therefore, they returned to Monterey County to sample tunicates again in 2018 and 2020.

A primary goal of this study was to verify whether snubnose sculpin, *O. triacis*, deposit egg masses in solitary tunicates, and potentially infer convergent evolution of egg deposition in tunicates on both sides of the Pacific in marine fishes. Specifically, the authors investigated whether parental species of egg masses found in the tunicates in the field were actually snubnose sculpin using genetic markers. Second, descriptions of host species and tunicate size were used to test whether the sculpin used specific species and size of hosts. Third, the authors compared ovipositor length of the snubnose sculpins with other ostracophilous sculpins inhabiting the Norhwestern Pacific (Awata *et al.*, 2019) to test whether ovipositor length is correlated with host size and morphology. Finally, the authors tested the hypothesis that egg deposition in solitary tunicates by sculpins has evolved convergently in species distributed in the Northeastern vs. the Northwestern Pacific. To test this, we reconstructed a molecular phylogeny using RNA-Seq from sculpins collected from both sides of the Pacific and estimated ancestral states of deposition of eggs in tunicates.

2 | MATERIALS AND METHODS

2.1 | Sample collections and measurements

Field collections were conducted in March 2018 and in March 2020, primarily on rocky reefs, at depths ranging from 0.5-17.0 m at five sites in Monterey County and at one site in Half Moon Bay, CA, USA (Figure 1), including the site where the authors found egg masses in tunicates in 2009. The collection periods coincide with the breeding season of common sculpin species of coastal California, including snubnose sculpins (Bolin, 1941; Morris, 1952; Hubbs, 1966; DeVlaming *et al.*, 1984; Grossman & DeVlaming, 1984; Petersen *et al.*, 2005; Pierce & Pierson, 1990; Ragland & Fischer, 1987; Snook, 1997).

To find egg masses in tunicates, 238 solitary tunicates were haphazardly collected using scuba on rocky reefs as well as on wharf pilings where they were abundant. All specimens were photographed with a ruler and their long diameter (D_L, to 0.1 mm) was later measured using the software ImageJ 1.52q. The D_L was used as an indicator of the body size of tunicates following Awata *et al.*, (2019). Tunicates were bisected vertically using a knife and photographed again with a ruler to measure the length from entrance of the atrial siphon to the nearest edge of the egg masses (defined as "egg mass position"; Awata *et al.*, 2019) when egg masses were discovered. The number of eggs (or hatched larvae in one clutch) in the host was counted, and the diameter of 10 eggs per egg mass was measured (to 0.01 mm) using photographs of eggs with a ruler and the software ImageJ 1.52q. Each egg mass was separately incubated at 15 °C until just before hatching before preservation in 99 % ethanol for genetic analyses.

The authors suspected that the parental species of egg masses deposited in solitary tunicates would be snubnose sculpins, but considered the possibility that the 10 other co-occurring sculpin species could also use solitary tunicates as hosts. Therefore, to identify the parental species of eggs found in tunicates using molecular markers, five sculpin species including snubnose sculpins were captured using scuba with a hand-net at five sites in Monterey County, and six sculpin species inhabiting tidepools were also collected by a hand-net at one site in Half Moon Bay (Figure 1; Supporting Information Table S1). Species identification was based on Miller and Lea (1976). Four of these 11 species are considered non-ostracophilous because females lay eggs on bottom substrates such as rocks and inside empty shells of barnacles; and the males guard multiple egg clutches from different females until hatching [*e.g., Artedius harringtoni* (Starks, 1896) and *Artedius fenestralis* (Girard, 1854): Ragland & Fischer, 1987; Petersen *et al.*, 2005; Lamb & Edgell, 2010; *Artedius corallinus* (Hubbs, 1926): S. Awata, T. Ito, Y. Koya, H. Munehara, unpubl. data; *Scorpaenichthys marmoratus* (Ayres, 1854): Feder et al., 1974; Lamb & Edgell, 2010]. It is unknown whether the other seven species are ostracophils. The authors used *cytochrome b* (*cytb*) sequences of these 11 species for identifying parental species of egg masses. All individuals were anaesthetised using 2-phenoxyethanol buffered with seawater, and a part of the pectoral fin was clipped from each fish and preserved in 99% ethanol for genetic analysis. Female snubnose sculpins (n = 20 individuals) were measured for standard length (L_S) to the nearest 0.1 mm using callipers. To examine the ovipositor morphology of the snubnose sculpins, the ovipositor was induced to its maximum extrusion length by gently pressing the abdominal region and photographed alongside a scale. The ovipositor length (L_O, to 0.1 mm) was measured using the software ImageJ 1.52q.

2.2 | Genetic identification of parental species of eggs in tunicates

To infer the parental species of the eggs in tunicates, eight embryos from four egg masses (two embryos per egg mass) and 22 fin-clip samples of 11 species of sculpins (Supporting Information Table S1) were genetically analysed following Awata *et al.*, (2019). Genomic DNA was extracted using a Gentra Puregene Tissue Kit (Qiagen, Hilden, Germany). The *cytb* locus was PCR amplified using the following primers: H15915- 5'-CAACGATCTCCGGTTT-3' and L14724- 5'-GTGACTTGAAAAACCA-3' (Schmidt & Gold, 1993). The PCR amplicons were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA). The purified PCR amplicons were sequenced on both strands by Macrogen Japan Corp. (Kyoto, Japan). Species identification of egg masses was inferred by comparison with the reconstructed phylogeny of the 11 sculpin species that occured in the collection area. The phylogenetic relationships were reconstructed using 1051 bp *cytb* sequences with MEGA version X (Kumar *et al.*, 2018; Stecher *et al.*, 2020), and the maximum likelihood method was employed using the Tamura Nei model settings and 1000 bootstrap replicates. The cabezon *S. marmoratus* was used as an outgroup following Smith and Busby (2014) and Betancur-R *et al.* (2017).

Sequences of the 11 sculpin species generated in this study are available on GenBank (Supporting Information Table S1).

2.3 | Phylogenetic analyses

The authors sampled testes or brains of 20 sculpin species collected from both sides of the Pacific (Supporing Information Table S1). These samples were preserved in RNA later solution (Thermo Fisher Scientific, Waltham, MA) and stored in -80˚C for phylogenetic analyses. The authors extracted the total RNA from the samples, using RNeasy Lipid Tissue Mini kit (Qiagen) following the manufacturer's protocol. RNA sequencing was performed on Illumina NovaSeq 6000 platform (Macrogen Japan Corp.) with 100 bp paired-end sequencing. Raw RNA data were quality checked and trimmed adapters and bases below $Q = 20$ by FaQCs ver. 1.34 (Lo & Chain, 2014), and short reads were *de novo* assembled by Trinity ver. 2.9.1 with the default parameters (Grabherr et al., 2011). The authors inferred orthologs of 20 species using Orthofinder ver. 1.0.6 (Emms & Kelly, 2019) with the default settings, and a total of 1,860,311bp of 1569 orthologs (Supporting Information Table S1) were used in the following analyses. The maximum likelihood phylogenetic tree was constructed by RAxML-NG ver. 1.0.2 (Kozlov et al., 2019) with the GTR+G+I model implemented. To evaluate the robustness of the node of ML tree, a bootstrap analysis was applied with 1000 replicates.

The authors also reconstructed ancestral states of deposition of eggs using Mesquite ver. 3.61 (Maddison & Maddison, 2019) with maximum likelihood method and model Mk1 model (Lewis, 2001). Two states for egg care patterns were identified: (0) egg care by male (non-ostracophilous) and (1) deposition of egg mass in tunicates or sponges (ostracophilous). Note that species with no data on egg care patterns were set as "unknown".

2.4 | Data analyses

Data analyses were carried out using SPSS ver. 25.0 (IBM Corp., Armonk, NY, USA). All tests were two-tailed and *P*-values <0.05 were considered statistically significant, or $P < 0.1$ when the sample size was small (*i.e., $n \leq 5$*). First, to test whether sculpins preferred specific size of tunicates, the authors performed *t*-test to compare average D_L and *F* test to compare variances between D_L of tunicates with and without eggs. Second, we examined whether ovipositor length of ostracophilous sculpins would reflect the species and size of the hosts used rather than the body size of sculpins (Awata *et al.*, 2019), L_S and L_O of snubnose sculpins were compared with those of the five ostracophilous sculpins (*F. ishikawae*, *F. osimae*, *P. marmoratus*, *P. zonostigma* and *V. centropomus*) from the Northwestern Pacific (data from Awata *et al.*, 2019), whose range in body size overlaps with snubnose sculpins, using *t*-test or Welch's *t*-test. Similarly, host size (D_L) and egg mass position within hosts were compared with the other five sculpins (Awata *et al.*, 2019) using *t*-test or Welch's *t*-test. In these tests, the probability of falsely rejecting the null hypothesis (*i.e.,* significance cutoff or alpha) was not adjusted because of the small sample size. Third, we examined whether the relative L_O of snubnose sculpins differed from other ostracophilous and non-ostracophilous sculpins from the Northwestern Pacific (all data from Awata *et al.*, 2019). To assess this, an analysis of covariance (ANCOVA) was used. The full model included $\log_{10}$-transformed L_O as the response variable, fish group categorized by host (five groups: snubnose sculpins, ostracophilous species that oviposit in solitary tunicates, colonial tunicates, sponges from the Northwestern Pacific and non-ostracophilous sculpins from the Northwestern Pacific) as a categorical factor, $\log_{10}$-transformed L_S as the covariate, and the interaction between fish group and $\log_{10}$-transformed L_S. As the interaction was non-significant ($P > 0.05$, see Results), a reduced model was constructed by removing the interaction term. Differences in $\log_{10} L_O$ among five groups of fishes were examined using Tukey's honest significant difference (HSD) post hoc test in the reduced model.

2.5 | Ethics statement

All field collections were conducted under the California Department of Fish and Wildlife scientific collection permit (Permit 2018 and 2020 to K.D.C., SC-13856 to S.A. and SC-192190004 to T.I.). Animal handling was conducted in accordance with the guidelines of Animal Care and Use Committees at Osaka City University, Osaka, Japan.

3 | RESULTS

3.1 | Host use and species identification of eggs in tunicates

Of the 238 tunicates collected, four species were identified (Table 1). *Ascidia ceratodes* (Huntsman, 1912) was the most prevalent on the wharf pilings and rocks at Stillwater Cove, CA (Figure 1). The other species identified at the study sites were rare (Table 1). Only 4 of the 238 solitary tunicates had egg masses and all were *A. ceratodes* (Figure 2a-c). The percent host occupancy among these samples was 1.86% (4/215; Table 1). The number of eggs per clutch was 171-346, and eggs were yellowish orange and approximately 1.0 mm in diameter (Supporting Information Table S2). Genetic identification of the parental species of egg masses found in solitary tunicates revealed that all four clutches were assigned to snubnose sculpins (Figure 2d; Supporting Information Figure S1). The authors examined whether female snubnose sculpins selected tunicates of specific body size. The mean and variance of host size were not different between used and unused *A. ceratodes*, although there appeared to be a tendency for female snubnose sculpins to use smaller *A. ceratodes* tunicates (Table 1; *t*-test, $t = 1.82$, $P = 0.07$; F test to compare two variances, $F = 3.64$, $P = 0.31$).

3.2 | Ovipositor length and host size

Ovipositor length (L_O) in female sculpins was correlated with standard length (L_S), and this relationship was distinct between species (Figure 3; full model, deposition site × log L_S: $F_{4,147} = 1.66$, $P = 0.16$; reduced model, log L_S: $F_{1,151} = 193.19$, $P < 0.0001$; deposition site: $F_{4,151} = 330.13$, $P < 0.0001$, Tukey's HSD post hoc test, $P < 0.05$ for all combinations). The ratio between L_O and L_S was 12.4% for the snubnose sculpin, 16.5–21.1% for the three other solitary tunicate-spawning sculpins, and 3.4% and 5.2% for colonial tunicate or sponge spawners (Table 2). Female snubnose sculpins had longer ovipositors (Figure 2e) compared to two sculpin species (*V. centropomus* and *P. marmoratus*) that deposit eggs in colonial tunicates and/or sponges (in both absolute and relative L_O: Table 2; Figure 3). In contrast, female snubnose sculpins had shorter L_O compared with three other sculpins that spawn in solitary tunicates (*F. osimae* and *F. ishikawae* and *P. zonostigma*, in both absolute and relative L_O; Table 2; Figure 3). Furthermore, host tunicates with larger body size (D_L) were used by sculpins with longer ovipositors, consistent with host specificity and co-evolution of ovipositor length and host morphology. Finally, egg mass position was also correlated with the sculpin L_O across the five sculpin species (Table 2).

3.3 | Ancestral state reconstruction of tunicate spawners

Ancestral state reconstruction indicated that male egg care (non-ostracophilous) was the ancestral state (Figure 4, probability = 95.8%). Furthermore, the occurrence of egg deposition in tunicates (ostracophilous) was found in two distantly related clades: the Northeastern (*O. triacis*) and the Northwestern Pacific (species of genera *Pseudoblennius* and *Furcina*).

4 | DISCUSSION

Egg masses deposited in the atrium of *A. ceratodes* tunicates were identified as eggs of the snubnose sculpin, *O. triacis*. Egg masses collected in 2009 (Supporting Information Figure S2) exhibited similar egg size (approximately 1 mm in diameter), egg number per clutch (a few hundred), and colouration (yellowish orange or orange) to the *O. triacis* egg masses collected in this study (see Supporting Information Table S2), suggesting similar parentage as these parameters are representative for this species. Because sculpins that oviposit in solitary tunicates have only been described from the Northwestern Pacific (Awata *et al.*, 2019; Nishida *et al.,* 2008), to the best of the authors' knowledge, this is the first record of tunicate spawning sculpins from the Northeastern Pacific. The reproductive strategy of using tunicates as egg hosts may be beneficial for sculpin development and fitness because of constant flow of filtered water in the atrium of tunicates and of protection from predators (Awata *et al.,* 2019; Hunter, 1969; Munehara, 1991; Spence & Smith 2013). This strategy would release female sculpins from extended parental care in species occurring in temperate and subarctic water, where embryonic periods are relatively long (16-19 days in *O. triacis*: Bolin, 1941; approximately 3 weeks in three genera *Furcina*, *Pseudoblennius* and *Vellitor*: Awata *et al.*, 2019; > 8 months in *B. cirrhosis*; Munehara, 1991).

A recent study has revealed that sculpins using solitary tunicates for oviposition have long ovipositors compared to sculpins using colonial tunicates and/or sponges, which have short ovipositors (Awata *et al.*, 2019). The authors found that snubnose sculpin females use solitary tunicates as their oviposition hosts, and similarly exhibit elongated ovipositors, that are much longer than the ovipositors of sculpins using colonial tunicates and/or sponges as egg hosts. Nonetheless, ovipositors of the snubnose sculpins were slightly shorter than those of similar body-sized sculpins that deposit eggs in solitary tunicates from the Northwestern Pacific. The authors propose that ovipositor length in ostracophilous sculpins, including snubnose sculpins, may be driven by host morphology based on the observation that ovipositor length was positively correlated with the length from entrance of the atrial siphon to the atrium where eggs are deposited. Indeed, female snubnose sculpins spawned in smaller tunicates with a shallower egg mass position than the solitary tunicate spawners in the Northwestern Pacific.

This adaptation of ovipositor length implies that snubnose sculpins exhibit host specificity. In this study, only *A. ceratodes* was observed as the oviposition host used by snubnose sculpins. As *A. ceratodes* was very abundant, and the other three species were uncommon at the study sites, it is difficult to assess host species preference of female snubnose sculpins. That is, it is likely that host availability could explain host utilization by female snubnose sculpins (Awata *et al.*, 2019; Kitamura, 2007; Reichard *et al.*, 2007). Nonetheless, the distribution pattern of the host tunicate widely overlaps with that of the snubnose sculpins, which may also imply the host specificity. *Ascidia ceratodes* is distributed along the Eastern Pacific coast from British Columbia, Canada, to Northern Chile, and is abundant in bays and harbours in northern and central California (Cañete *et al.*, 2019; Lambert & Lambert, 1998; Van Name, 1945), and snubnose sculpins occur in a sub-set of that range, from British Columbia, Canada, to Baja California (Koya *et al.,* 2011; Lamb & Edgell, 2010; Miller & Lea, 1976). The snubnose sculpin is common from northern to central California, and is particularly abundant in Monterey Bay (Snook, 1997; this study). It would be interesting to investigate host use by the snubnose sculpins and their ovipositor length in regions where potential hosts are more diverse, and not dominated by *A. ceratodes*.

The authors collected 10 commonly occurring sculpin species in addition to snubnose sculpins at depths of 0.1-17.0 m off of Monterey County and Half Moon Bay. Of these 10 species, four are known to be non-ostracophilous (*A. harringtoni*, *A. fenestralis*, *A. corallinus* and *S. marmoratus*: see Section 2.1, “Sample collections”), but it is unknown whether the other six species are ostracophils. IGA is an important predictor of ostracophily in sculpins, sea ravens and poachers (Abe & Munehara, 2009; Awata *et al.*, 2019; Koya *et al.*, 2015; Munehara, 1991). Indeed, snubnose sculpins are a copulatory species that exhibit IGA (Bolin, 1941; Koya *et al.*, 2011), and the authors found that male snubnose sculpins have a relatively long genital papilla (contrary to what was reported in Bolin, 1941, 1944 and Buser *et al.*, 2017), which is normally contracted in the abdomen and extruded only when the abdominal region was gently pressed, similar to elongated ovipositors in females (Supporting Information Figure S3). Of the 10 species of sculpins collected and genetically analysed in this study (see Supporting Information Figure S1 and Table S1), five species, such as *A. harringtoni*, *Clinocottus analis* (Girard, 1858), *Clinocottus. recalvus* (Greeley, 1899), *Oligocottus maculosus* Girard, 1856, and *Oligocottus. snyderi* Greeley, 1898, are also copulatory IGA species (Buser *et al.*, 2017; DeMartini & Patten, 1979; Hubbs, 1966; Koya *et al.*, 2011; Morris, 1952, 1956; Ragland & Fischer, 1987; Stein, 1973), but *A. harringtoni* are a non-ostracophilous species. The other four sculpin species inhabiting tidepools (genus *Clinocottus* and *Oligocottus*) have the potential to deposit their eggs in live invertebrates, but their spawning sites in the field have not been characterized, although deposited egg clutches have been collected in aquaria for *C. recalvus* (Morris, 1952) and *O. maculosus* (Atkinson, 1939; Pierce & Pierson, 1990; Stein, 1973).

The morphological and molecular phylogeny of the marine sculpins shows convergent evolution of reproductive modes on both sides of the Pacific (Knope, 2013; Smith & Busby, 2014; Yabe 1985), from non-copulating ancestors to copulatory species exhibiting IGA (Abe & Munehara, 2009; but see Buser *et al.,* 2017). Furthermore, ostracophilous sculpins may have evolved from non-ostracophilous sculpins (Abe & Munehara, 2009; Knope, 2013; Koya, *et al.* 2011; Smith & Busby, 2014; Yabe, 1985). The phylogenetic analysis of this study using 1.86 Mbp RNAseq data of 20 sculpin species supports these two hypotheses. More importantly, ancestral state reconstruction indicated that male egg care was the ancestral state and egg deposition in tunicates occurs in two distantly related clades in the Northeastern (*O. triacis*) and the Northwestern Pacific (species of genera *Pseudoblennius* and *Furcina*). Because the ancestral reproductive modes differ in the sister taxa of ostracophilous species in the Northeastern or the Northwestern Pacific, utilization of solitary tunicates as deposition hosts has arisen multiple times independently via convergent evolution in species endemic to the Northeastern or the Northwestern Pacific.

In summary, the authors have reported that snubnose sculpins use the solitary tunicate *A. ceratodes* as egg brooding hosts in the Northeastern Pacific, and that ovipositor length and host size are commensurate with clutch position in this species. Recent DNA barcoding studies have found that several marine fishes deposit their eggs inside invertebrates (*e.g.,* Awata *et al.*, 2019; Gardner *et al.*, 2016; Matsuzaki *et al.*, 2020) and externally on the body of invertebrates (Busby *et al.*, 2006; Levin & Rouse, 2020). Nonetheless, only limited data on the behavioural and morphological adaptations of parasites and hosts are available. To the best of the authors' knowledge, this is the first study to investigate adaptive traits associated with reproductive mode in snubnose sculpin in a comparative and phylogenetic context.

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AUTHOR CONTRIBUTIONS

S.A. and H.M. conceived and designed the study; all authors conducted field sampling; S.A. and T. I. performed morphological, genetic and phylogenetic data analyses; the manuscript was written by S.A., with significant contributions from K.D.C. All authors contributed critically to the drafts and gave approval for publication.

SUPPORTING INFOTMATION

Additional supporting information may be found in the online version of the article at the publisher’s website.

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FIGURE CAPTIONS

FIGURE 1 Location of five sampling sites on the coast of Monterey, Pacific Grove, and Pebble Beach, Monterey County, CA, USA. Point Pinos: 36° 38′ 22.32″ N, 121° 55′ 24.00″ W; Lovers Point: 36° 37′ 37.53″ N, 121° 54′ 43.62″ W; Breakwater: 36° 36′ 33.91″ N, 121° 53′ 30.17″ W; Share Island: 36° 36′ 31.08″ N, 121° 52′ 43.92″ W; Stillwater Cove: 36° 33′ 56.87″ N, 121° 56′ 34.45″ W. Sculpins inhabiting tidepools were also collected at Pillar Point: 37° 29′ 40.76″ N, 122° 29′ 53.15″ W, Half Moon Bay, San Mateo County, CA, USA.

FIGURE 2 Photographs of host tunicates, deposited eggs, and the ostracophilous sculpin. (a) *Ascidia ceratodes*, the tunicates utilised by the snubnose sculpin (*Orthonopias triacis*) as oviposition hosts. (b) Opened *Ascidia ceratodes* with eyed eggs of snubnose sculpin (indicated by the white arrowhead). (c) Enlarged photograph of eyed eggs of snubnose sculpin. (d) Female snubnose sculpin. (e) Ovipositor of snubnose sculpin.

FIGURE 3 The relationship between $\log_{10}$ standard length (L_S) and $\log_{10}$ ovipositor length (L_O) of snubnose sculpin (*Orthonopias triacis*) and the ostracophilous and non-ostracophilous sculpins from the Northwestern Pacific (all data other than the data of snubnose sculpins from Awata *et al.*, 2019). *Circles*, *triangles*, and *squares* denote the species that used solitary tunicates, colonial tunicates and sponges, and rock surfaces as deposition sites, respectively. Regression lines for different types of hosts are from the reduced model of ANCOVA.

FIGURE 4 | Phylogenetic analysis of the evolution of deposition of egg mass in tunicates or sponges (ostracophilous) in marine sculpins. The maximum likelihood phylogenetic tree of the 20 sculpin species collected on the shores in central California (the Northeastern Pacific) and in Japan (the Northwestern Pacific) as reconstructed by RAxML-NG ver. 1.0.2 based on 1860311bp from 1569 orthologs, with the settings of GTR+G+I model and 1000 bootstrap replicates. Values at nodes are bootstrap percentages. The probability of each ostracophilous occurring at ancestral nodes is indicated with pie charts at the nodes: black: deposition of egg mass in tunicates or sponges (ostracophilous); white: egg care by male (non-ostracophilous); and grey: unknown. Although parental care and spawning site of *Icelinus cavifrons* was unknown, this species was categorized into non-ostracophilous, since it was non-copulating species. Outlined and non-outlined scientific names indicate the species collected in the Northeastern and the Northwestern Pacific, respectively. The presence or absence of copulation (Cop.), parental care patterns (Par. care), spawning site (Spa. site), and the references (Ref.) are also shown. NC: Non-copulating species; C: Copulating species; SW: seaweed; Sol tun: Solitary tunicate; Col tun: colonial tunicate. 1: Feder *et al.*, 1974; 2: Lamb & Edgell, 2010; 3: S. Awata, T. Ito, Y. Koya, H. Munehara, unpub. Data; 4: Ito & Awata, 2019; 5: Ragland & Fisher, 1987; 6: Petersen *et al.*, 2005; 7: Bolin, 1941; 8: Koya *et al.*, 2011; 9: this study; 10: Hubbs, 1966; 11: Stein, 1973; 12: Morris, 1956; 13: Tsuruoka *et al.*, 2006; 14: Koya *et al.*, 1993; 15: Munehara *et al.*, 1989; 16: Koya *et al.*, 2002; Awata *et al.*, 2019; 18: Koya *et al.*, 2015; 19: Nishida *et al.*, 2008.

TABLE 1 Body size of collected tunicates and host use by the snubnose sculpin *Orthonopias triacis*

	Long diameter of tunicates (D_L), mean ± SD (range, *n*)	
Tunicate species	**Without any egg masses**	**With sculpin egg masses**
Ascidia ceratodes	32.9 mm ± 9.1 (14.7-57.5 mm, *n* = 211)	24.6 mm ± 4.8 (17.4-27.1 mm, *n* = 4)
Cnemidocarpa finmarkiensis	20.5 mm ± 5.0 (13.7-25.8 mm, *n* = 5)	-
Halocynthia igaboja	35.6 mm ± 8.3 (29.1-49.0 mm, *n* = 5)	-
Styela montereyensis	113.9 mm ± 15.7 (88.4-139.6 mm, *n* = 13)	-

TABLE 2 Comparisons in standard length, ovipositor length, and long diameter and egg mass position of hosts between snubnose sculpins (*Orthonopias triacis*) and five ostracophilous sculpins that oviposit in tunicates or sponges in Japan (all data from Awata *et al.*, 2019)

	Solitary tunicate spawners				**Colonial tunicate or sponge spawners**	
Spawner	***Orthonopias triacis***	***Furcina osimae***	***F. ishikawae***	***Pseudoblennius zonostigma***	***Vellitor centropomus***	***P. marmoratus***
Standard length (L_S, mm)	53.6 ± 7.8 (20)	49.4 ± 6.7 (5)$^{\mathrm{ns}}$	52.1 ± 7.8 (9)$^{\mathrm{ns}}$	85.6 ± 11.6 (14)***	59.9 ± 7.6 (22)*	85.9 ± 14.6 (7)***
	40.2-68.2	44.5-61.1	40.0-67.0	68.0-110.0	50.3-73.9	64.7-106.6
Ovipositor length (L_O, mm)	6.6 ± 1.5 (20)	8.1 ± 1.8 (5)$^{\#}$	9.8 ± 1.7 (9)***	18.5 ± 7.5 (14)***	3.2 ± 0.7 (22)***	2.9 ± 0.6 (7)***
	3.0-9.2	5.2-10.1	7.1-12.2	8.7-37.7	2.2-4.7	2.0-3.8
L_O/L_S x 100 (%)†	12.4 ± 2.6 (20)	16.5 ± 3.7 (5)	19.2 ± 4.1 (9)	21.1 ± 6.2 (14)	5.2 ± 0.7 (22)	3.4 ± 0.7 (7)
	7.5-18.0	11.6-21.6	13.6-26.3	12.2-34.3	4.0-6.8	2.8-4.5
Host tunicate						
Long diameter (D_L, mm)	24.6 ± 4.78 (4)	38.9 ± 11.8 (3)$^{\#}$	17.5 (1)	98.3 ± 17.5 (13)***	-	-
	17.4-27.1	32.0-52.5		76.3-125.9		
Egg mass position (mm)	3.7 ± 0.2 (4)	6.0 ± 1.1 (3)**	-	20.4 ± 5.8 (11)***	2.3 ± 1.8 (13)*	2.1 ± 1.8 (4)$^{\mathrm{ns}}$
	3.2-4.1	5.0-7.1		13.3-33.7	0.0-5.3	0.2-4.5

Mean ± standard deviation (SD), sample sizes in parentheses, and ranges are shown.

ns: $P > 0.1$; #: $0.05 < P < 0.1$; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ by *t*-test or Welch's *t*-test.

†Statistical tests were not performed.

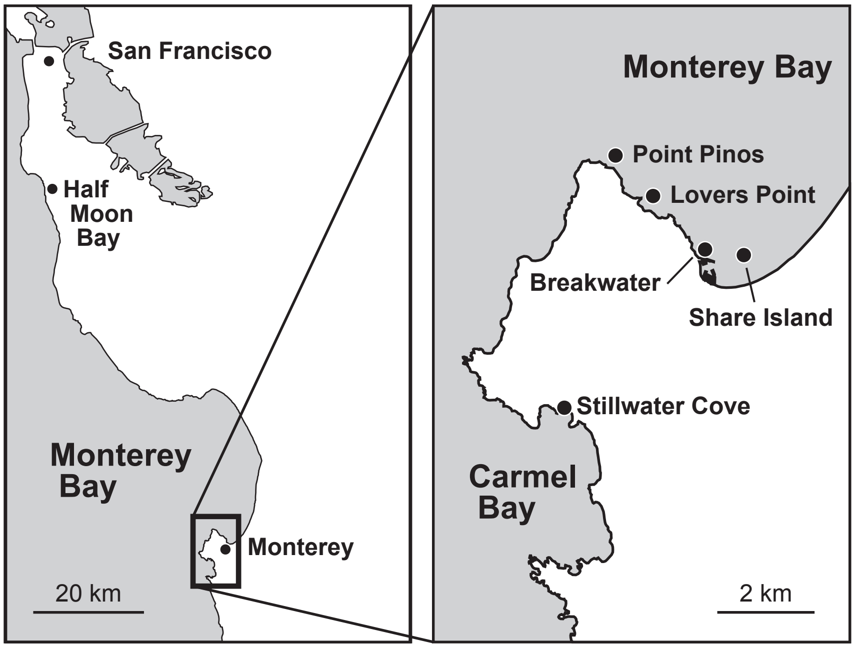

San Francisco
Half Moon Bay
Monterey Bay
Monterey
20 km
Monterey Bay
Point Pinos
Lovers Point
Breakwater
Share Island
Stillwater Cove
Carmel Bay
2 km

FIGURE 1

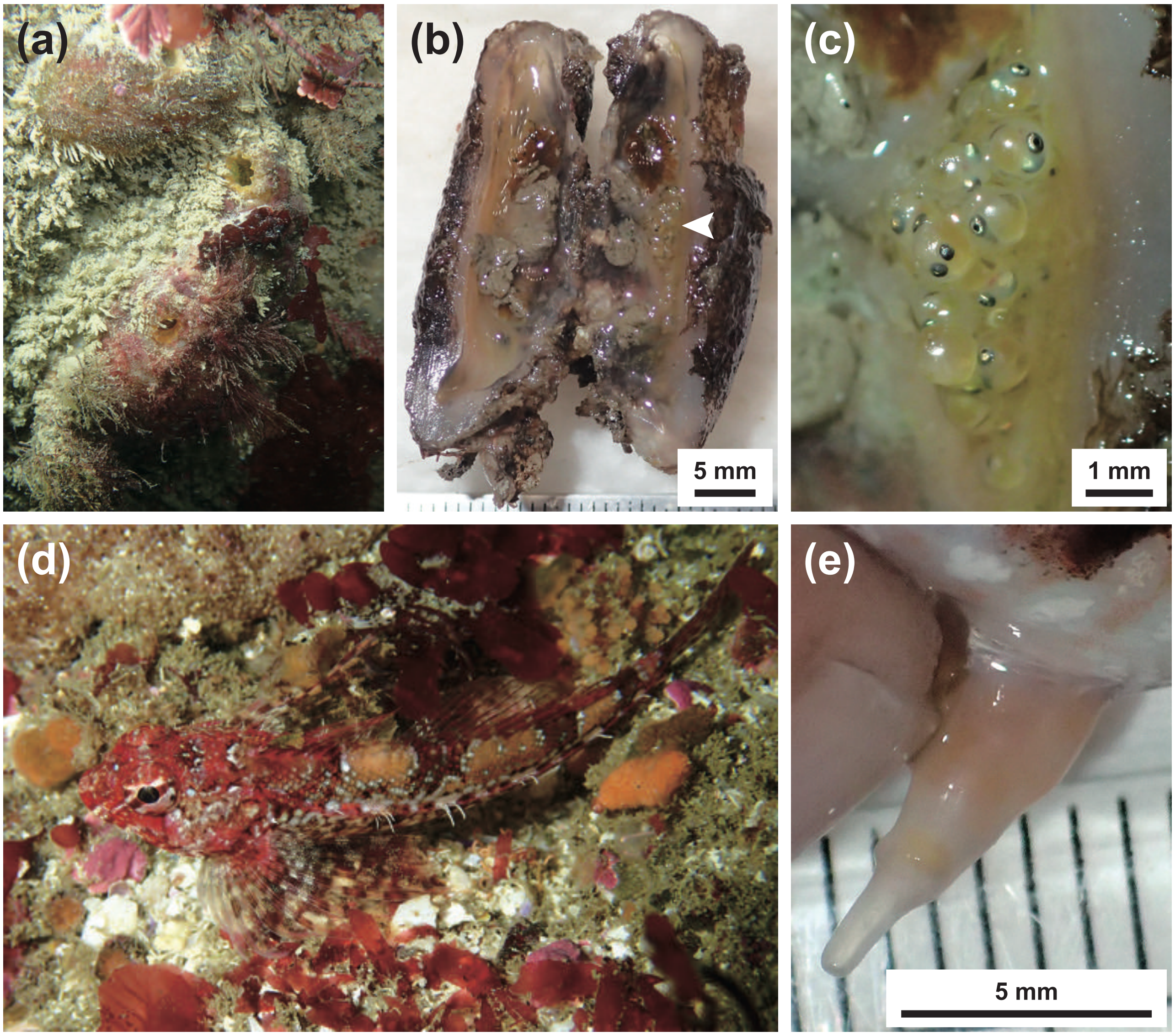


FIGURE 2

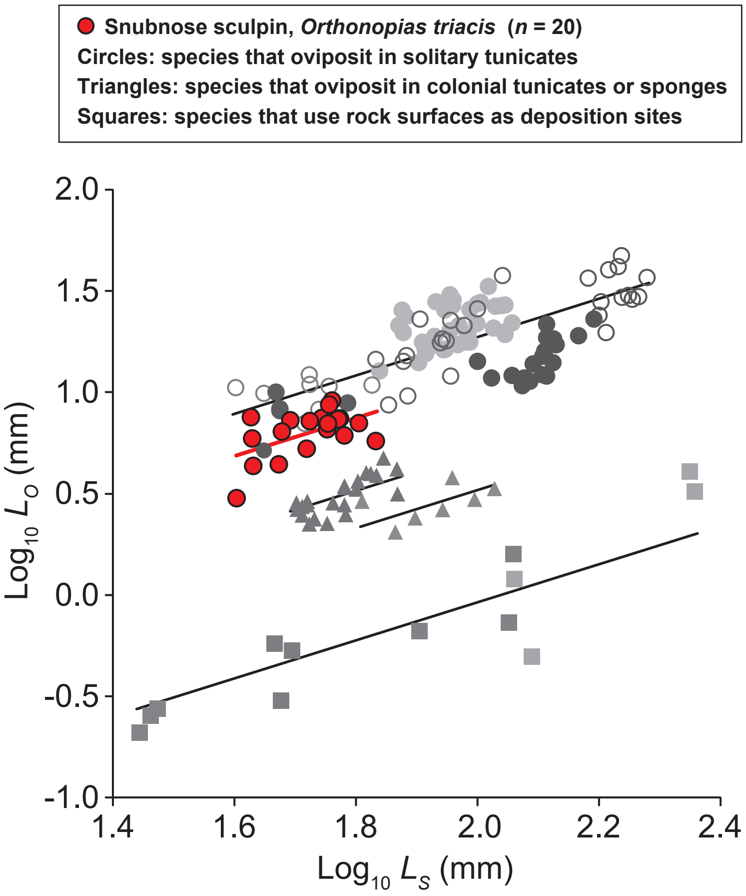
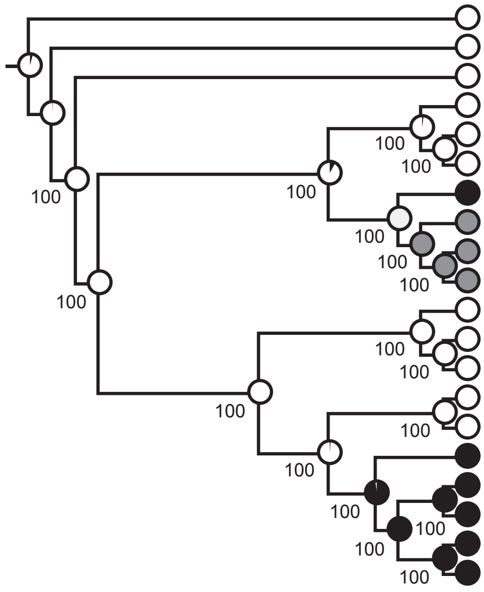

● Snubnose sculpin, Orthonopias triacis (n = 20)
Circles: species that oviposit in solitary tunicates
Triangles: species that oviposit in colonial tunicates or sponges
Squares: species that use rock surfaces as deposition sites
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
Log10 LO (mm)
1.4
1.6
1.8
2.0
2.2
2.4
Log10 LS (mm)

FIGURE 3



Species name	Cop.	Par. care	Spa. site	Ref.
Scorpaenichthys marmoratus	NC	Male	Rock	1,2,3
Gymnocanthus intermedius	NC	Male	Rock	3
Icelus mororanis	NC	Male	Rock SW	3,4
Artedius harringtoni	C	Male	Rock	2,3,5,6
Artedius corallinus	NC	Male	Rock	3
Artedius lateralis	NC	Male	Rock	2,3,6
Orthonopias triacis	C	No care	Sol tun	3,7,8,9
Clinocottus analis	C	-	-	3,10
Oligocottus maculosus	C	-	-	3,8,11
Oligocottus snyderi	C	-	-	3,12
Icelinus cavifrons	NC	-	-	3
Icelinus pietschi	NC	Male	Rock	3,13
Icelinus japonicus	NC	Male	Rock	3
Bero elegans	C	Male	Rock	3,14
Alcichthys alcicornis	C	Male	Rock	3,15,16
Furcina osimae	C	No care	Sol tun	3,17
Pseudoblennius marmoratus	C	No care	Sponge	3,4,17
Vellitor centropomus	C	No care	Col tun	3,17,18
Pseudoblennius cottoides	C	No care	Sol tun	3,8,17
Pseudoblennius percoides	C	No care	Sol tun	3,17,19

FIGURE 4

TABLE S1 Individual identity, scientific name, collection site, collection date, depth, and GenBank accession number of sculpins used for this study.

Ind. ID	Scientific name	Collection site	Depth	Collection date	Accession No.
Artedius corallinus-20	*Artedius corallinus*	Share Island, Monterey, Monterey County, CA, USA	6 m	05 March 2018	LC546698
Artedius corallinus-22	*Artedius corallinus*	Off Lovers Point, Pacific Grove, Monterey County, CA, USA	9 m	06 March 2018	LC546699
Artedius harringtoni-13	*Artedius harringtoni*	Off Lovers Point, Pacific Grove, Monterey County, CA, USA	9-12 m	06 March 2018	LC546700
Artedius harringtoni-14	*Artedius harringtoni*	Off Lovers Point, Pacific Grove, Monterey County, CA, USA	9 m	06 March 2018	LC546701
Artedius lateralis-8	*Artedius lateralis*	Pillar Point, Half Moon Bay, San Mateo County, CA, USA	<0.5 m	02 March 2018	LC546702
Clinocottus analis-1	*Clinocottus analis*	Pillar Point, Half Moon Bay, San Mateo County, CA, USA	<0.5 m	02 March 2018	LC546703
Clinocottus analis-2	*Clinocottus analis*	Pillar Point, Half Moon Bay, San Mateo County, CA, USA	<0.5 m	02 March 2018	LC546704
Clinocottus recalvus-1	*Clinocottus recalvus*	Pillar Point, Half Moon Bay, San Mateo County, CA, USA	<0.5 m	02 March 2018	LC546705
Icelinus cavifrons-1	*Icelinus cavifrons*	Share Island, Monterey, Monterey County, CA, USA	15 m	05 March 2018	LC546706
Icelinus cavifrons-2	*Icelinus cavifrons*	Share Island, Monterey, Monterey County, CA, USA	15 m	05 March 2018	LC546707
Leptocottus armatus-1	*Leptocottus armatus*	Pillar Point, Half Moon Bay, San Mateo County, CA, USA	2 m	08 March 2018	LC546708
Oligocottus maculosus-6	*Oligocottus maculosus*	Pillar Point, Half Moon Bay, San Mateo County, CA, USA	<0.5 m	08 March 2018	LC546709
Oligocottus maculosus-7	*Oligocottus maculosus*	Pillar Point, Half Moon Bay, San Mateo County, CA, USA	<0.5 m	08 March 2018	LC546710
Oligocottus snyderi-5	*Oligocottus snyderi*	Pillar Point, Half Moon Bay, San Mateo County, CA, USA	<0.5 m	02 March 2018	LC546711
Oligocottus snyderi-8	*Oligocottus snyderi*	Pillar Point, Half Moon Bay, San Mateo County, CA, USA	<0.5 m	02 March 2018	LC546712
Orthonopias triacis-16	*Orthonopias triacis*	Share Island, Monterey, Monterey County, CA, USA	17 m	05 March 2018	LC546713
Orthonopias triacis-17	*Orthonopias triacis*	Share Island, Monterey, Monterey County, CA, USA	17 m	05 March 2018	Same sequence as LC546713
Orthonopias triacis-22	*Orthonopias triacis*	Off Lovers Point, Pacific Grove, Monterey County, CA, USA	10-14 m	06 March 2018	Same sequence as LC546713
Orthonopias triacis-28	*Orthonopias triacis*	Off Lovers Point, Pacific Grove, Monterey County, CA, USA	10-14 m	06 March 2018	Same sequence as LC546713
Orthonopias triacis-29	*Orthonopias triacis*	Off Lovers Point, Pacific Grove, Monterey County, CA, USA	10-14 m	06 March 2018	Same sequence as LC546713
Scorpaenichthys marmoratus-4	*Scorpaenichthys marmoratus*	Share Island, Monterey, Monterey County, CA, USA	13 m	05 March 2018	LC546714
Scorpaenichthys marmoratus-6	*Scorpaenichthys marmoratus*	Off Lovers Point, Pacific Grove, Monterey County, CA, USA	10 m	06 March 2018	LC546715
Artedius corallinus-2020-1	*Artedius corallinus*	Lovers Point, Pacific Grove, Monterey County, CA, USA	2 m	18 February 2020	DRR317847
Artedius harringtoni-2020-3	*Artedius harringtoni*	Breakwater, Monterey, Monterey County, CA, USA	5 m	02 March 2020	DRR317845
Artedius lateralis-2020-2	*Artedius lateralis*	Off Edward F. Ricketts State, Monterey, Monterey County, CA, USA	5 m	05 March 2020	DRR317846
Clinocottus analis-2020-3	*Clinocottus analis*	Pillar Point, Half Moon Bay, San Mateo County, CA, USA	<0.5 m	24 May 2020	DRR317849
Icelinus cavifrons-2020-1	*Icelinus cavifrons*	Share Island, Monterey, Monterey County, CA, USA	15 m	05 March 2020	DRR317852
Oligocottus maculosus-2020-1	*Oligocottus maculosus*	Pillar Point, Half Moon Bay, San Mateo County, CA, USA	<0.5 m	10 January 2020	DRR317850
Oligocottus snyderi-2019-6	*Oligocottus snyderi*	Pillar Point, Half Moon Bay, San Mateo County, CA, USA	<0.5 m	24 December 2019	DRR317851
Orthonopias triacis-2020-1	*Orthonopias triacis*	Breakwater, Monterey, Monterey County, CA, USA	3 m	18 February 2020	DRR317848
Scorpaenichthys marmoratus-2020-2	*Scorpaenichthys marmoratus*	Pillar Point, Half Moon Bay, San Mateo County, CA, USA	<0.5 m	04 April 2020	DRR317842
Alcichthys alcicornis-2019-3	*Alcichthys alcicornis*	Usujiri, Hakodate, Hokkaido, Japan	10 m	15 April 2019	DRR317856
Bero elegans-2019-1	*Bero elegans*	Usujiri, Hakodate, Hokkaido, Japan	7 m	17 April 2019	DRR317855
Furcina osimae-201812-6	*Furcina osimae*	Hiraiso, Hitachinaka, Ibaraki, Japan	<0.5 m	22 December 2018	DRR317857
Gymnocanthus intermedius-2018-1	*Gymnocanthus intermedius*	Usujiri, Hakodate, Hokkaido, Japan	30-50 m	06 December 2018	DRR317843
Icelinus japonicus-2019-1	*Icelinus japonicus*	Mushizaki, Sado, Niigata, Japan	11-17m	16 January 2019	DRR317854
Icelinus pietschi-2019-2	*Icelinus pietschi*	Mushizaki, Sado, Niigata, Japan	11-17m	16 January 2019	DRR317853
Icelus mororanis-2019-3	*Icelus mororanis*	Usujiri, Hakodate, Hokkaido, Japan	9-13 m	17 April 2019	DRR317844
Pseudoblennius cottoides-Hiroshima-1	*Pseudoblennius cottoides*	Takehara, Hiroshima, Japan	3 m	09 November 2017	DRR317861
Pseudoblennius marmoratus-201812-1	*Pseudoblennius marmoratus*	Hiraiso, Hitachinaka, Ibaraki, Japan	<0.5 m	22 December 2018	DRR317858
Pseudoblennius percoides-Hiroshima-1	*Pseudoblennius percoides*	Takehara, Hiroshima, Japan	3 m	09 November 2017	DRR317860
Vellitor centropomus-2020-1	*Vellitor centropomus*	Mushizaki, Sado, Niigata, Japan	5-10 m	17 November 2020	DRR317859

TABLE S2 Results of genetic identification of parental species of egg masses deposited in solitary tunicates. Sampling date, locality, depth, water temperature (WT), number of eggs in hosts, number of hatched larvae, the mean egg diameter, egg (yolk) colouration and number of young analysed for genetic species identification are shown.

No	Egg mass ID	Fish species	Host ID	Host species	Date	Locality	Depth	WT	Number of eggs	Number of hatched larvae	Mean egg diameter (*n*)	Egg (yolk) colouration	Number of analysed young
1	STE-01	*Orthonopias triacis*	ST-076	*Ascidia ceratodes*	03-Mar-20	Stillwater Cove, Pebble Beach, Monterey County, CA, USA	0.5-3.0 m	13.0°C	171	-	1.00 mm (10)	Yellowish orange	2
2	STE-02	*Orthonopias triacis*	ST-077	*Ascidia ceratodes*	03-Mar-20	Stillwater Cove, Pebble Beach, Monterey County, CA, USA	0.5-3.0 m	13.0°C	-	93	-	-	2
3	STE-03	*Orthonopias triacis*	ST-095	*Ascidia ceratodes*	06-Mar-20	Stillwater Cove, Pebble Beach, Monterey County, CA, USA	0.5-3.0 m	14.6°C	346	-	0.95 mm (10)	Yellowish orange	2
4	STE-04	*Orthonopias triacis*	ST-212	*Ascidia ceratodes*	06-Mar-20	Stillwater Cove, Pebble Beach, Monterey County, CA, USA	0.5-3.0 m	14.6°C	173	-	1.02 mm (10)	Yellowish orange	2

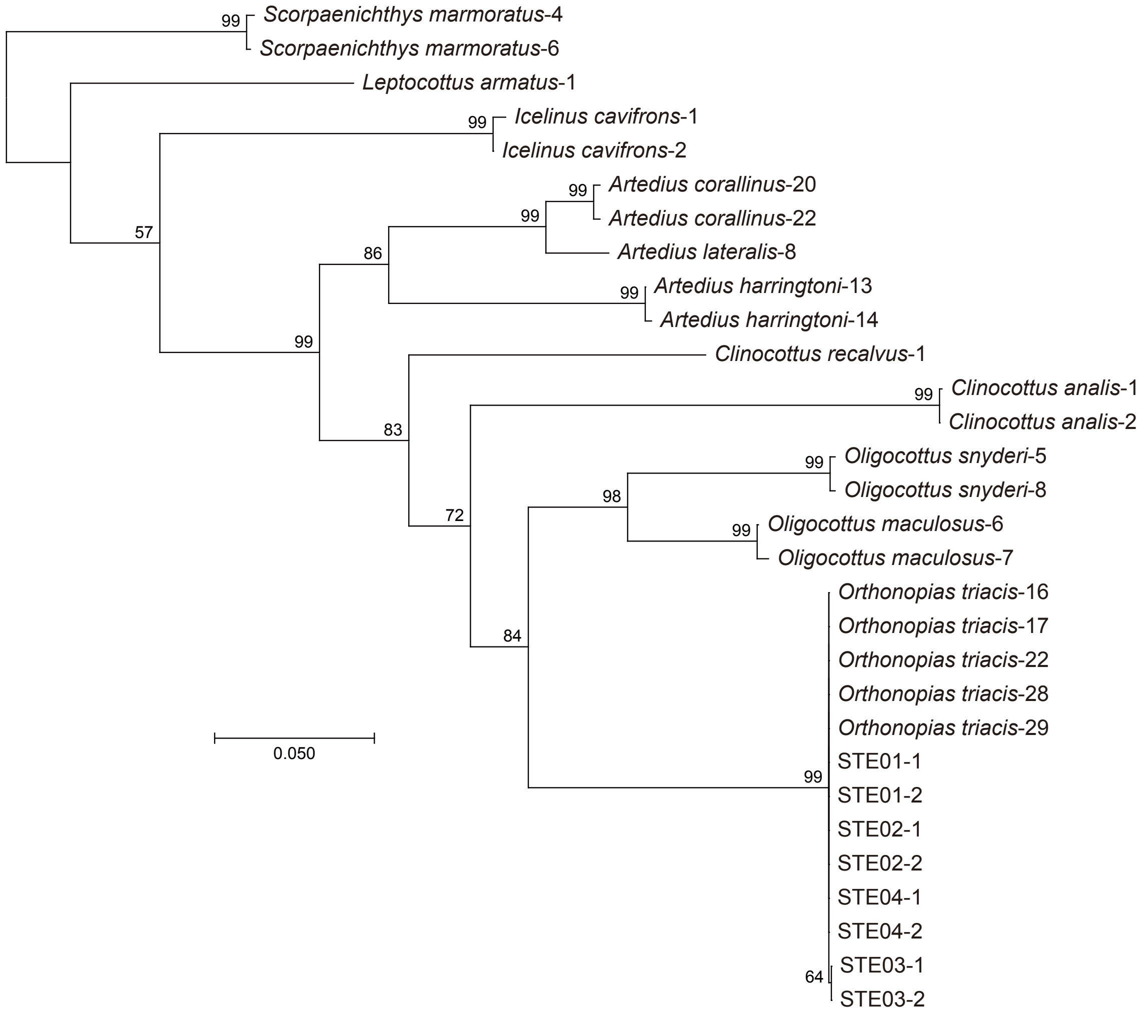


FIGURE S1 Maximum likelihood phylogeny of the 11 sculpin species and eight embryos from four egg masses collected on the shore in central California as reconstructed by MEGA version X using 1051 bp *cytb* sequences, with the settings of the Tamura Nei model and 1000 bootstrap replicates. Values at nodes are bootstrap percentages.

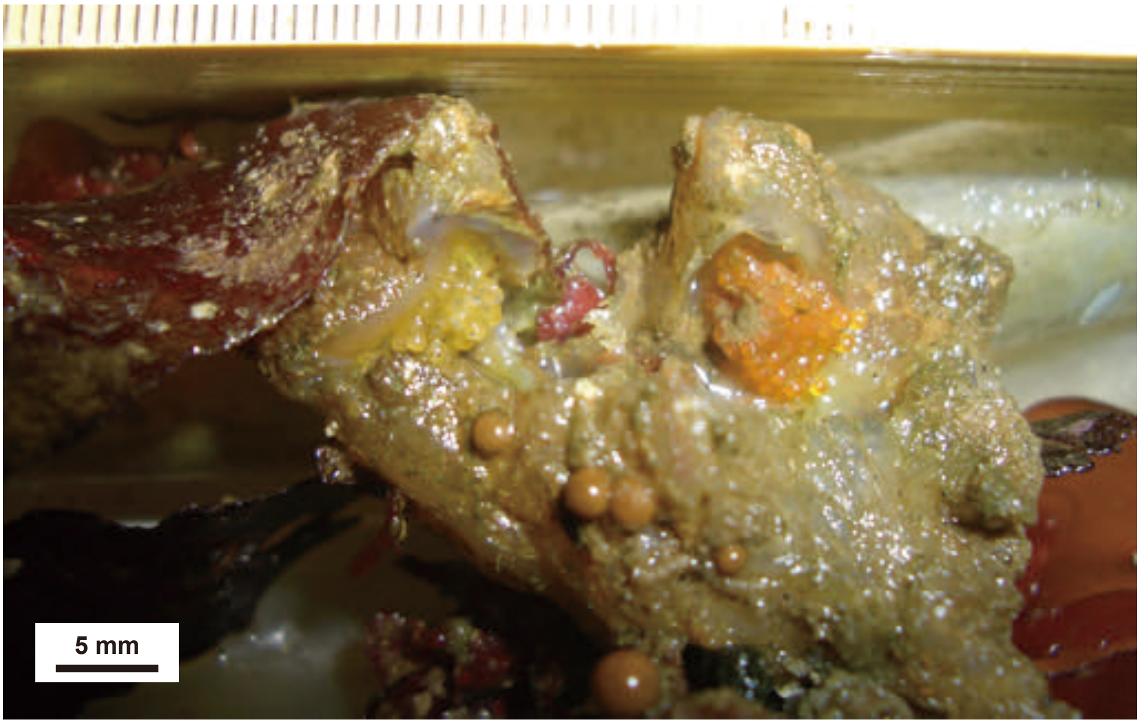


FIGURE S2 A photograph of two egg masses found in a tunicate during the field study in 2009. These egg masses possess some characteristics of eggs of snubnose sculpin (*Orthonopias triacis*): c.a. 1 mm in diameter of eggs with yellow and orange colouration.

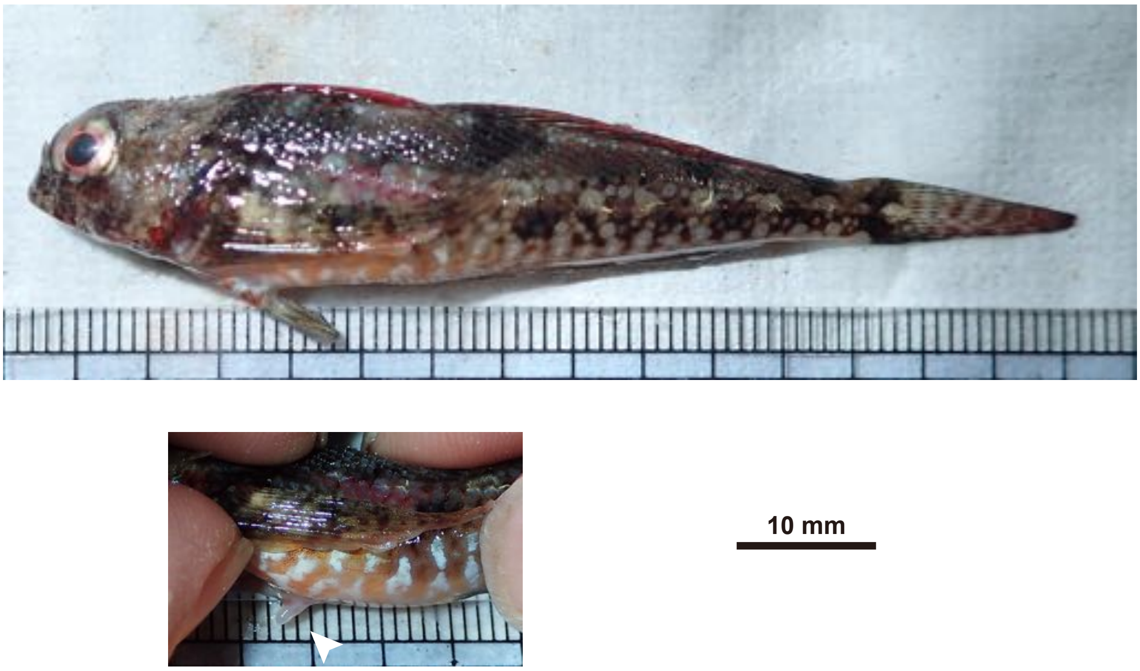


FIGURE S3 A photograph of the male snubnose sculpin (*Orthonopias triacis*) and its genital papilla (indicated by the white arrowhead).