



BRILL



brill.com/ctoz

Insights into a putative polychaete-gastropod symbiosis from a newly identified annelid worm that predated upon *Conus ermineus* eggs

Matías L. Giglio | ORCID: 0000-0002-6357-9772

Corresponding author,

School of Biological Sciences, University of Utah, 257 South 1400 East, Salt Lake City, UT 84112-0840, USA

matias.giglio@utah.edu

Paula Flórez Salcedo | ORCID: 0000-0002-2109-8612

Corresponding author,

Department of Neurobiology, University of Utah, 20 South 2030 East, Salt Lake City, UT 84112, USA

paula.florez@neuro.utah.edu

Maren Watkins | ORCID: 0000-0002-4127-1961

School of Biological Sciences, University of Utah, 257 South 1400 East, Salt Lake City, UT 84112-0840, USA

Baldomero Olivera | ORCID: 0000-0003-4556-1410

School of Biological Sciences, University of Utah, 257 South 1400 East, Salt Lake City, UT 84112-0840, USA

RECEIVED: 26 JULY 2022 | REVISED AND ACCEPTED: 24 NOVEMBER 2022 | PUBLISHED

ONLINE 17 FEBRUARY 2023; PUBLISHED IN ISSUE 3 APRIL 2023

EDITOR: B.W. HOEKSEMA

Abstract

Cone snails are a diverse group of venomous marine gastropods that have dioecious reproduction and internal fertilization resulting in egg deposition inside capsules. However, the observational studies conducted on their spawning behaviour and egg masses have left many open questions. Here, we analyse egg masses from a specimen of *Conus ermineus* kept in captivity for over 17 years. We present the first detailed description of the morphological features of the egg capsules and eggs (e.g., egg shape, size,

and colour). The analysis of these capsules led us to the identification of a dinophilid worm (Polychaeta: Dinophilidae) living inside the snail egg capsules and likely feeding upon the snail eggs. This is the first report of such behaviours among dinophilids. Our analysis suggests that these worms belong to a new species, here described as *Dimorphilus oophagus* sp. nov., supported by both molecular and morphological data. Finally, we discuss the possibility of a putative symbiotic relationship between the worm and the snail.

Keywords

Dimorphilus – oophagia – oviposition

Introduction

Cone snails (genus *Conus* Linnaeus, 1758) are a large and diverse lineage of marine gastropods with over 750 extant species (WoRMS, consulted in October 2022) distributed worldwide in tropical and subtropical regions (Olivera et al., 2014). They are well known for their cone-shaped shells with strikingly beautiful colour patterns, many of which are highly desired among collectors (Terlau & Olivera, 2004). Cone snails have gained scientific attention due to the biomedical potential of their venom. Every species of *Conus* produces a unique venom cocktail comprising 100–400 small active peptides known as conotoxins (Olivera et al., 2017). The snails use harpoon-like radular teeth to inject these toxins into their prey, consisting mainly of worms, other molluscs, or fish (Terlau & Olivera, 2004).

All species of *Conus* are presumed to be dioecious and have internal fertilization (Kohn, 1961a, 2012). Females lay eggs surrounded by an albuminous material and packed in horny capsules called egg capsules. Often, these egg capsules form clusters called egg masses (Kohn, 1961a). The egg capsules show interspecific variability, but often they are flattened elongated pouches with an attaching stalk and a thin membrane, the operculum, located at

the distal end. The operculum is progressively digested during larval development to finally allow the exit of the larvae (Kohn, 1961a). Every individual egg capsule can contain from tens to thousands of rounded-to-oval eggs of 125 μm to 1 mm in diameter (Kohn, 2012). Most studies on cone snail reproductive structures used egg masses obtained from the field or laid shortly after the specimens were transferred to laboratory water tanks (e.g., Cruz & Olivera, 1978; D'Asaro, 1970a, b; Gonçalves et al., 2017; Kohn, 1961a; Lebour, 1945; Natarajan, 1957; Nybakken, 1967; Zehra & Perveen, 1991). Here we report an intriguing case of egg deposition of a specimen of *Conus ermineus* Born, 1778 after 17 years of captivity.

Conus ermineus inhabits the Atlantic Ocean (fig. 1A) (Olivera et al., 2014; Puillandre et al., 2014) and, together with the Eastern Pacific *Conus purpurascens* Sowerby, 1833, belongs to the subgenus *Chelyconus*, a clade of fish-hunting snails that has evolved independently from other piscivorous Indo-Pacific species (fig. 1A, B) (Duda & Palumbi, 2004; Olivera et al., 2014; Puillandre et al., 2014, Ramiro et al., 2022). Both species originated from the same lineage after a relatively recent speciation event by vicariance related to the emergence of the Isthmus of Panama (Puillandre et al., 2014). Studies on the oviposition of *C. ermineus* are scarce, but their egg masses

and capsules have been described for specimens from Colombia and Venezuela (Bandel, 1976; Penchaszadeh, 1984).

In 2004, we received a specimen of *C. ermineus* collected from the Gulf of Mexico and kept it in a laboratory tank over the past 18 years. In June 2021, after 17 years of captivity, the specimen laid a set of egg masses, some of them infested by annelid worms apparently feeding upon the recently laid cone snail eggs. The worms belonged to the genus *Dimorphilus* Worsaae et al., 2019 which includes meiobenthic, free-living, microscopic polychaetes having a poorly defined segmentation, highly marked sexual dimorphism, and a small genome (Martín-Durán et al., 2021; Worsaae et al., 2019, 2021). To date, the genus includes only two species, *Dimorphilus gyrociliatus* (Schmidt, 1857), from Italy, and *Dimorphilus kincaidi* (Jones & Ferguson, 1957), from the USA (Schmidt, 1857; Jones & Ferguson, 1957; Worsaae, 2019, 2021). The populations of *D. gyrociliatus* reported from Japan and China most probably belong to different lineages, thus requiring further analysis to be characterized (Worsaae, 2019).

Here we describe the general characteristics of the egg capsules of *C. ermineus* and fully describe and illustrate the associated worms living inside and feeding on the eggs as belonging to a new species of *Dimorphilus*.

Materials and methods

Specimen source of egg masses

The Gulf of Mexico specimen of *C. ermineus* was kept in a 100-liter tank filled with water containing Instant Ocean® Sea Salt (United Pet Group, Inc., Ohio, USA) at room temperature and under a 12 h:12 h light:dark cycle. It was fed regularly with goldfish, *Carassius auratus*, raised in separate tanks. A specimen of *C. purpurascens* collected in 2008 off the coast of

Panama was introduced into the same tank, which they share since then. After some mating behaviours (including active chasing and repetitive mounting), in June 2021, the specimen of *C. ermineus* (which measured 66 mm in total length; fig. 2A) deposited several egg masses on the surface of the coral-like rocks of the tank.

Egg masses, egg capsules, and eggs

Egg masses and capsules were removed from the tank within 72 h after oviposition using forceps and transferred to Petri dishes, rinsed in Hanks' Balanced Salt solution (HBSS), photographed, and dissected to obtain the internal content. A few capsules were left in the tank to be monitored. The area close to the stalk of each capsule was repeatedly pressed to open the operculum and expulse the content. Both capsules and eggs were photographed using a digital camera adapted to a stereomicroscope (AmScope, Irvine, CA, USA). The egg contents were pooled and stored either in HBSS (Gibco, Life Technologies Corp., NY, USA) or RNAlater (Invitrogen, Thermo Fisher Scientific, Vilnius, Lithuania) at -80°C . The content of the capsules having living worm-like organisms were separated, photographed, and fixed in 70% ethanol. The worms were sorted and observed under an inverted microscope Eclipse Ti-U (Nikon Instruments Inc., Melville, NY, USA) connected to a Zyla sCMOS camera (Andor, Concord, MA, USA) for morphological identification. Some worms were kept in aerated artificial sea water supplemented with triturated commercial seaweed for further observations. The type materials of the new species have been deposited at the Natural History Museum of Utah (UMNH).

Additionally, we prospected for living worms inside the water tank sucking ~ 20 L of water using a hose (7-mm inner diameter) connected to a 10-mL serological pipette from

the water column, the tank filter system, rock crevices, and in between the sand-like substrate. Samples were pooled and concentrated using a vacuum filtration apparatus with a 0.45 μm MCE filter membrane (Merk Millipore Ltd., Tullagreen, Ireland). The concentrated samples were analysed following the same protocols as the egg capsule contents.

Genetic analyses

Genomic DNA was extracted from both the egg masses infested by the worms and free-swimming worms using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, Minnesota, USA), following the standard protocol provided by the manufacturer.

Ten nanograms of egg DNA and 2.5 ng worm DNA were used as templates for polymerase chain reaction (PCR) using oligonucleotide primers corresponding to the polychaete mitochondrial cytochrome oxidase I gene (COI) barcode region modified from Lobo Arteaga et al. (2015). The primers included cloning tails designed for the USER-friendly™ cloning system (New England Biolabs, Beverly, Massachusetts, USA). Forward primer: 5' ggagaca/dU/TCHACAAAYCAYAARGAYATHGGWAC 3'. Reverse primer: 5' gggaag/dU/ACTTCWGGRTGWCCRAARAAYCA 3'. The PCR amplification protocol consisted of an initial denaturation (95°C, 60 sec) followed by 5 cycles of denaturation (95°C, 20 sec), annealing (45°C, 20 sec) and extension (72°C, 30 sec), then 35 cycles of denaturation (95°C, 20 sec), annealing (54°C, 20 sec) and extension (72°C, 30 sec). The PCR products (720 bp) were subsequently isolated from a 0.9% LE agarose gel, then excised and recovered using the Roche High Pure PCR Product Purification Kit (Sigma-Aldrich, St. Louis, Missouri, USA) following the manufacturer protocol, and annealed to pNEB206A vector according to the User-Friendly Cloning Kit Protocol (New

England Biolabs, Beverly, Massachusetts, USA) following the manufacturer protocol. The resulting cloning products were transformed into DH5 α competent cells (ThermoFisher Scientific, Waltham, Massachusetts, USA). Nucleic acid sequences were then determined by automated sequencing (Core Sequencing Facility, University of Utah, USA). The sequences recovered from both the contaminated egg and the worms belonged to *Dimorphilus* worms. These sequences were aligned and determined to a shared 99.8% identity. The sequences of *Dimorphilus* COI-encoding clones were aligned to that of *C. ermineus* COI (GenBank: FJ937340.1) and were determined to share only a 70.7% identity. The *Dimorphilus* COI sequence was deposited in GenBank (OM868217). Attempts to recover the COI sequence of *Conus* from eggs were unsuccessful.

Phylogenetic analysis

Phylogenetic analysis was performed using the COI barcode sequences of *Dimorphilus* identified from the infested eggs of *C. ermineus* and from isolated worms, along with previously reported mtCOI sequences of other Dinophilidae (table 1). Multiple sequence alignment was performed using MAFFT version 7 (Kato et al., 2017), maximum likelihood tree reconstruction was performed using IQ tree (Nguyen et al., 2015), consisting of best-fit model GTR+F+I+G4 using ModelFinder (Kalyaanamoorthy et al., 2017) and ultrafast bootstrap approximation with 1000 replicates (Minh et al., 2013). The tree was visualized using iTOL version 6.4 (Letunic & Bork, 2021) with midpoint rooting.

Fluorescence staining and image processing

Individual adult worms were immunohistochemical stained targeting acetylated alpha-tubulin to identify the surface ciliary arrangement. Specimens were fixed using

TABLE 1 Genbank accession numbers of the COI gene sequences used in this study

Genus	Species	COI
<i>Protodorvillea</i>	<i>kefersteini</i>	KF808171.1
<i>Phylo</i>	<i>foetida</i>	MT499170.1
<i>Trilobdrilus</i>	<i>axi</i>	MK896709.1
<i>Trilobdrilus</i>	<i>heideri</i>	MK896666.1
<i>Trilobdrilus</i>	<i>itoi</i>	AB924371.1
<i>Dinophilus</i>	<i>vorticoides</i>	MK896710.1
<i>Dinophilus</i>	<i>taeniatus</i>	MK896707.1
<i>Dinophilus</i>	sp. KW591a	MK896697.1
<i>Dinophilus</i>	<i>gardineri</i>	MK896704.1
<i>Trilobdrilus</i>	<i>nipponicus</i>	LC009442.1
<i>Trilobdrilus</i>	sp. KW745	MK896705.1
<i>Trilobdrilus</i>	<i>windansea</i>	MG588093.1
<i>Trilobdrilus</i>	<i>ellenscrippsae</i>	MG588094.1
<i>Dimorphilus</i>	<i>gyrociliatus</i> _Italy	MK896663.1
<i>Dimorphilus</i>	<i>gyrociliatus</i> _Japan	LC545951.1
<i>Dimorphilus</i>	<i>cf. kincaidi</i>	MK896714.1
<i>Dimorphilus</i>	<i>oophagus</i> sp. nov.	OM868217
<i>Dimorphilus</i>	<i>gyrociliatus</i> _China	MK896685.1
<i>Diurodrilus</i>	<i>subterraneus</i>	KC790350.1
<i>Mesonerilla</i>	<i>intermedia</i>	MK896665.1

a 4% paraformaldehyde solution in PBS (1.5 mM NaH₂PO₄, 8.1 mM Na₂HPO₄, 140 mM NaCl, 2.7 mM KCl, pH 7.4) overnight at 4°C. Then, tissues were permeabilized by incubating with 0.25% Tween-100 for 30 min at room temperature, followed by 30 min incubation in blocking solution containing 10% donkey serum (Jackson ImmunoResearch Inc., PA, USA, #241112) and 30 mM glycine (Sigma-Aldrich, St. Louis, Missouri, USA) in PBS. Monoclonal anti-acetylated tubulin antibody (Sigma-Aldrich, USA; clone 6-11B-1T-7451) diluted 1:5000 was used. The primary antibody was visualized using goat anti-mouse IgG conjugated with Alexa Fluor-647 (Invitrogen, Thermo Fisher Scientific, Vilnius, Lithuania, #A21236) diluted 1:1000. The specimens were transferred to a 2-chamber cell imaging slide (Eppendorf, Enfield, CT, USA) and images on

an inverted confocal microscope SP8 (Leica Microsystems, Wetzlar, Germany) using a 10 × /1.0-NA objective. Specimens were imaged at a size of 1024 × 1024 × 0.65 pixels (XYZ). All images were acquired using Nikon NIS-element platform.

Results

Egg masses, egg capsules, and eggs of

Conus ermineus

We found 36 capsules, most of which organized in three egg masses with 8, 10, and 12 capsules. Six additional capsules individually deposited were also found in the tank (fig. 2B). The capsules were rectangular with a convex and a concave side. The convex side had pronounced transverse ridges giving the lateral

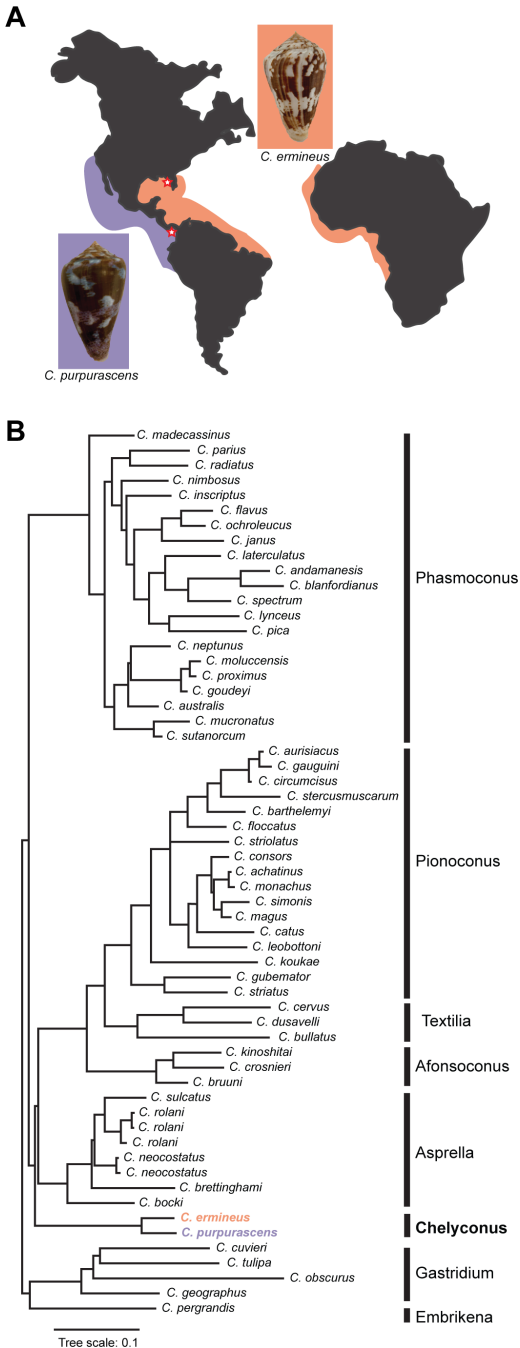


FIGURE 1 Distribution and phylogeny of cone snails. A: Distribution of *Conus ermineus* (orange) and *Conus purpurascens* (purple), modified from Monnier et al. (2018); Red stars: collection sites. B: Phylogenetic tree of fish-hunting species of *Conus*, modified from Ramiro et al. (2022).

edges a festooned look (fig. 2C, D). The concave side was smooth, with transverse ridges barely noticeable on the borders. The capsule measured 11.5–13.8 mm high (average = 12.36 mm), 7–8.3 mm wide (average = 7.74 mm), and 2.1–3 mm thick (average = 2.57 mm). The operculum was elongated, occupied most of the top edge, and was surrounded by small folds from both walls (fig. 2D). The capsules were found either independently or in clusters of up to six arranged in rows, being held together by a confluent basal plate, with the concave side of a capsule towards the convex side of the adjacent one. The capsule walls were semi-transparent off-white, allowing easy visualization of the internal eggs (fig. 2C). Most capsules contained from hundreds to a few thousand eggs, although some were empty. The eggs were spherical, pale orange, and measured 150 μm in diameter, on average (fig. 2E). The eggs inside the capsules left in the tank did not develop, showing traces of decomposition after one month.

Egg capsules infested with worms

Despite having the transparent, white walls apparently intact, approximately half of the capsules did not contain pale orange eggs, but a dusky grey matter (fig. 3A) consisting of worms surrounded by the same mucous material as the eggs (fig. 3B). These capsules also contained few eggs, some of which showing alterations such as reduced size, non-rounded shape, and empty shells. The worms were alive and swam in a circular motion, while some had a substance inside the anterior digestive track showing a pale orange colour similar to the intact cone snail eggs observed in other capsules, clearly distinguishable from the worms' oocytes (fig. 3C, D).

Molecular identification of the worms

Our genetic analysis is consistent with previous molecular analysis and supports the

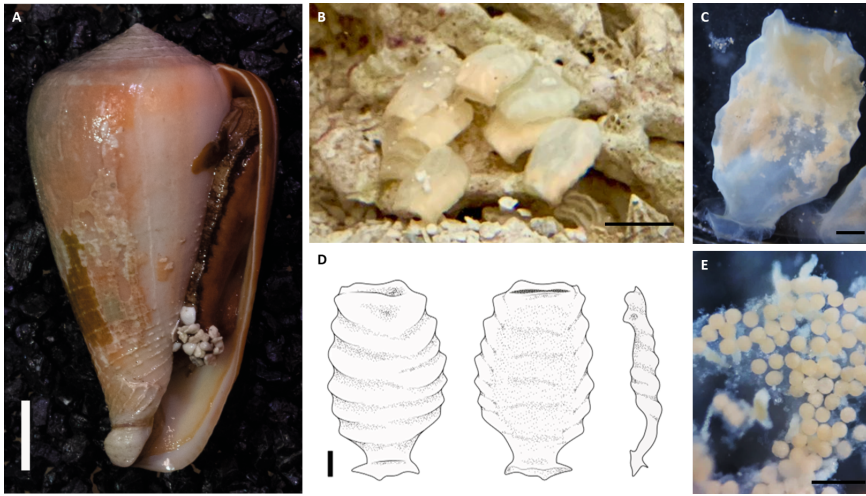


FIGURE 2 Egg capsules of *Conus ermineus*. A: Living specimen retracted inside the shell. Photo by Samuel Espino. B: Egg masses attached to coral-like rocks. C: Detail of an egg capsule. D: Schematic representation of an egg capsule; left = convex wall, center = concave wall, right = lateral view. E: Detail of the eggs. Scale bars: A = 1 cm, B = 10 mm, C = 2 mm, D = 2 mm, E = 500 μm .

monophyly of *Dimorphilus* (fig. 4A), while showing that our sequences corresponded to a new species of *Dimorphilus*. This species is recovered as a sister taxon of *D. gyro-ciliatus*_China and forms a branch with the clade ((*D. gyro-ciliatus*_Italy, *D. gyro-ciliatus*_Japan) *D. kincaidi*) (fig. 4A). The COI sequence has <85.5% similarity with the previously reported sequences (fig. 4B).

Examination of the tank water, revealed the presence of different microscopic invertebrates, including copepods and polychaetes (none of them belonging to the new species). The specimens of the new species kept in captivity did not survive beyond 48 h.

Taxonomic account

Phylum Annelida Lamarck, 1802

Class Polychaeta Grube, 1850

Family Dinophilidae Macalister, 1876

Genus *Dimorphilus* Worsaae, Kerbl, Vang & Gonzalez, 2019

Type species. *Dimorphilus gyro-ciliatus* (Schmidt, 1857).

Generic diagnosis. Fully agrees with that in Worsae et al. (2019).

Dimorphilus oophagus sp. nov.

Zoobank: urn:lsid:zoobank.org:act:5F04BFD0-4062-42E9-8BD8-48A979C4FA07

Material examined. Holotype: adult female collected inside the egg capsules of *C. ermineus* living in captivity at the University of Utah in June 2021 by Matías L. Giglio and Paula Flórez Salcedo, fixed and preserved in 70% ethanol (Voucher# UMNH.ann.0001770); Paratype: same collection data and preservation as holotype (Voucher# UMNH.ann.0001771).

Diagnosis. Based on females; prostomium with two ciliary tufts projecting anteriorly, and two ciliary bands, second one complete; eyespots rounded.

Description. *Adults:* Females body hyaline, cylindrical, plump shape 512.8 – 692.31 μm long (mean = 615.35 μm), with six segments between the prostomium and pygidium (fig. 5A, E). Prostomium with two well-defined,

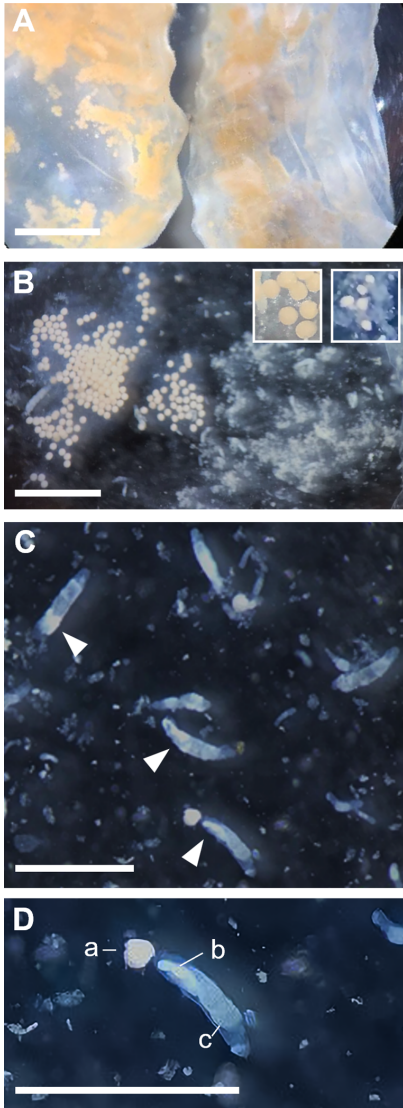


FIGURE 3 Contents of the capsules of *Conus ermineus*. A: External appearance of non-infested (left) and infested (right) capsules. B: Contents of capsules filled with eggs (left) and infested with worms (right). *Insets*: comparison of unaffected (left) and affected (right) eggs. C: Specimens of *Dimorphilus oophagus* sp. nov.; arrowheads = egg-like contents. D: Enhanced resolution image of a specimen of *D. oophagus* sp. nov. eating *C. ermineus* eggs. a = cone snail egg, b = cone snail egg-like content in the gut of the worm, c = worm oocytes. Scale bars: B = 2 mm, C = 1 mm, D = 1 mm.

round-shaped, pigmented eyespots, two ciliary tufts projecting terminally, and two sets of cilia organized in two bands (an incomplete band between eyespots and anterior end, and a complete band between eyes and mouth) (fig. 5A, C, E). Each trunk segment with a central transversal ciliary band (fig. 5 A, D, E). Pygidium short, conical, lacking cilia.

Prominent muscular pharynx well defined located at level of segment 1. Two thirds of mid body cavity full of pale white/grey oocytes (37.7 – 88.9 μm in diameter). Males not observed.

Juvenile: body elongated, slender (fig. 5B), always smaller than the adult forms. Identical to adult females except for having less segments and lacking oocytes.

Type locality. Salt Lake City, Utah, USA.

Etymology: the specific epithet “*oophagus*” is the Greek form for “egg eater,” and refers to the atypical egg-feeding behaviour of the species.

Remarks. *Dimorphilus oophagus* sp. nov. can be clearly distinguished from the other two species of the genus by a combination of traits including ciliary arrangement and eyespot shape (table 2). The three species have no males in their original descriptions, presumably due to the difficultness in finding them, as they are dwarf and have short lifespans, characteristic of other species of the genus. Unfortunately, we do not have enough material of females or juveniles, to further analyse the morphology of these specimens (i.e., using electron microscope or histology).

Nomenclatural acts. The electronic version of this article as a PDF represents published work according to the International Commission on Zoological Nomenclature (ICZN), and thus the new species name is regarded as published. This published work and the Nomenclatural acts it contains have been registered in ZooBank with the LSID urn:lsid:zoobank.org:pub:A1646078-E274-4D44-B661-EC071B8E56F2 for the publication

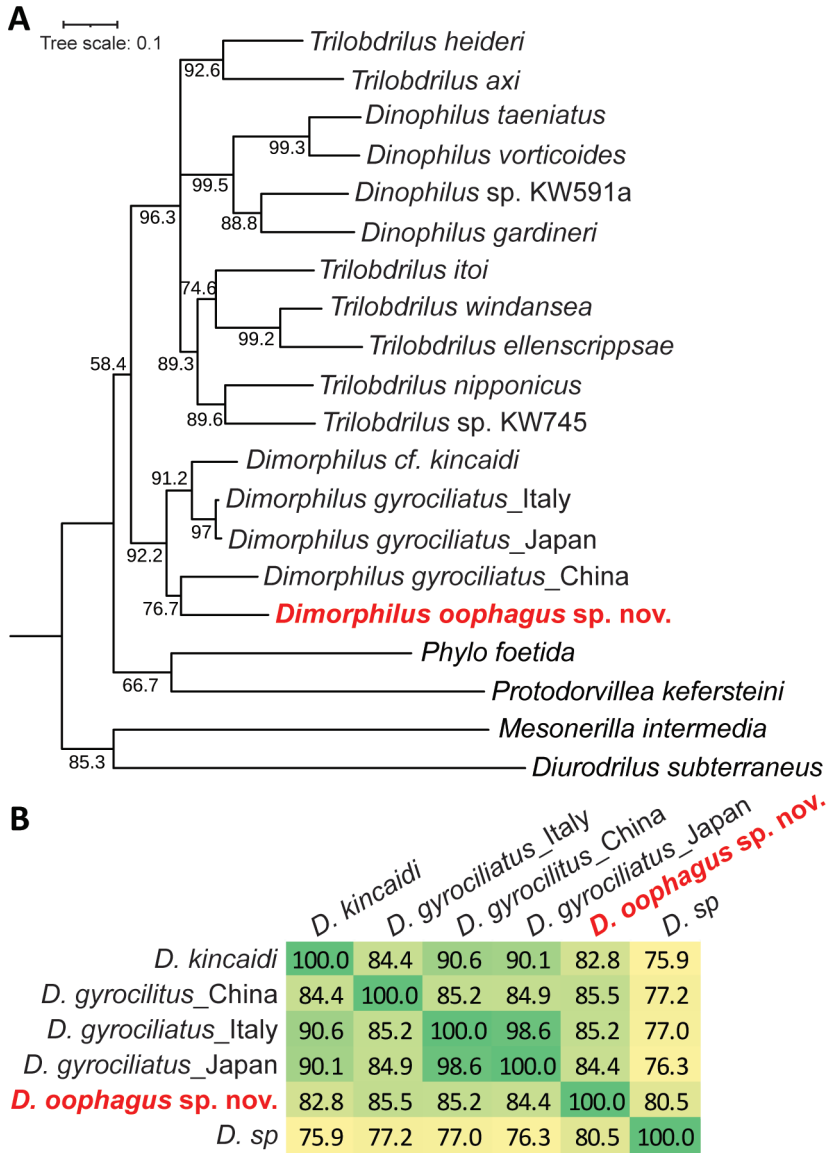


FIGURE 4 Phylogenetic relationships of *Dimorphilus oophagus* sp. nov. A: Maximum likelihood phylogenetic tree; numbers in nodes indicate bootstrap support. Branches with support values under 50 were collapsed. B: COI sequence identity among the species and/or populations of *Dimorphilus*.

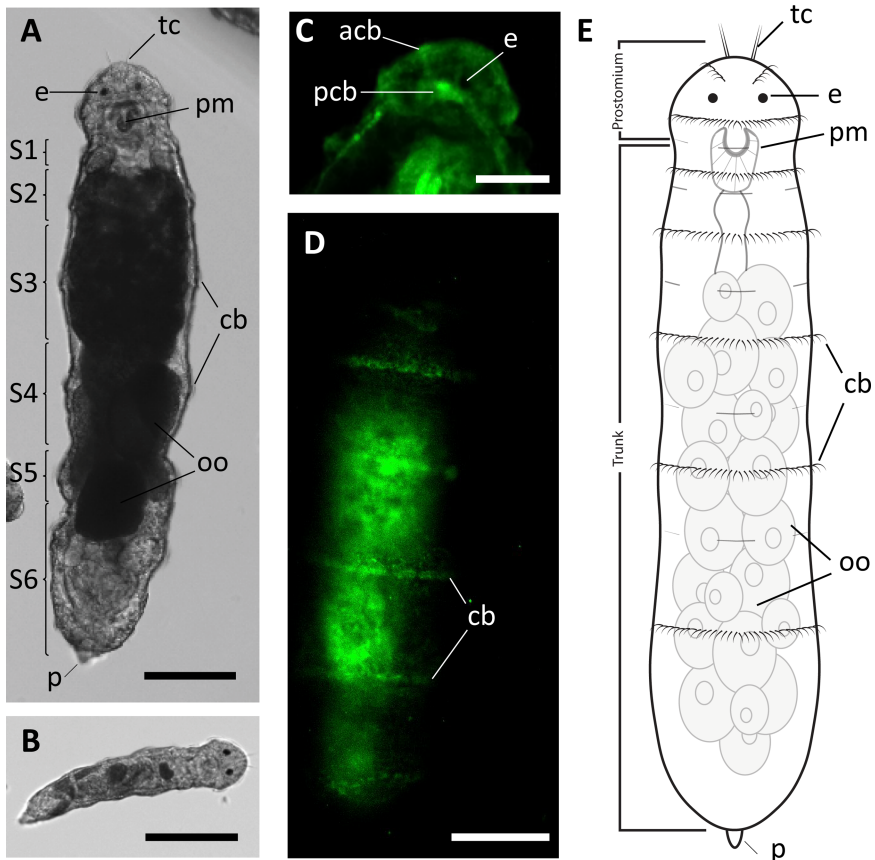


FIGURE 5 *Dimorphilus oophagus* sp. nov. A: Adult female, dorsal view; cb: ciliary bands, e: eyespot, oo: intra-celomic oocytes, pm: pharyngeal musculature, p: pygidium, tc: tuft of cilia. S1–S6 = segment 1–6 B: Juvenile female. C: Immunohistochemistry showing the prostomial ciliary arrangement of an adult female. Acb = anterior ciliary band, pcb = posterior ciliary band D: Immunohistochemistry showing the segmental ciliary bands of an adult female. E: Reconstruction of an adult female of *Dimorphilus oophagus* sp. nov. Scale bars: A = 100 μ m, B = 100 μ m, C = 50 μ m, D = 100 μ m.

and urn:lsid:zoobank.org:act:5F04BFD0-4062-42E9-8BD8-48A979C4FA07 for the species.

Discussion

Reproduction of *Conus ermineus*

Studies on cone snail reproductive strategy, especially on their egg masses, are relatively scarce and discontinuous over time, as usually they do not lay eggs in captivity long after being collected, except *C. geographus* Linnaeus, 1758 which spawned after a year (Cruz & Olivera, 1978). Our specimen of *C. ermineus* spawned

after almost two decades of captivity, and only after sharing a tank for more than five years with a specimen of *C. purpurascens*. Although mating performances were observed, we are unaware of whether internal fecundation occurred. Since these two species are closely related and have diverged relatively recently (Puillandre et al., 2014), cross-reproduction cannot be entirely discarded. However, the “eggs” found in the tank showed no evidence of development after one month following the oviposition, which could be explained either by being unfertilized (i.e., oocytes) or by the existence of post-fertilization reproductive

TABLE 2 Comparison among *Dimorphilus* species

Trait	<i>D. oophagus</i> sp. nov.	<i>D. gyrotiliatus</i>	<i>D. kinkaidi</i>
Ciliary tufts	One pair projecting anteriorly	Two pairs	One pair projecting anteriorly
Ciliary band anterior to the eyes	Discontinuous	Discontinuous	Continuous
Ciliary band posterior to the eyes	Continuous	Discontinuous	Discontinuous
Eyespots	Rounded	Kidney-shaped	Rounded
Traverse ciliary band in trunk segments	Single, central in all segments	Single, central in all segments	Single central in segments 1 to 3. Displaced posteriorly in segment 4, then 2 bands resulting from fusion of segments 5 and 6
Pygidium	Not ciliated	Not ciliated	Ciliated
Reference	This paper	Schmidt, 1857	Jones, 1957

barriers. Unfortunately, we were not able to obtain egg DNA, preventing us from deciphering its genetic identity. However, our data suggest that cone snail oviposition can be stimulated by some mating performances (e.g., mounting) even if occurring between different species and thus deriving into internal fertilization.

The egg capsules found in the tank were morphologically similar to those previously reported for this species (Bandel, 1976; Penchaszadeh, 1984). They were also organized in rows, as observed by Bandel (1976) and Penchaszadeh (1984). Capsules of this species were also found in overlapping layers, indicating a certain level of intraspecific variation (Penchaszadeh, 1984). Capsules may contain a highly variable number of “eggs” (from hundreds to thousands, even within the same egg mass), and rounded like most species of *Conus*, but showing a diameter closer to the lower range for the genus. Egg size has a strong direct relationship with prehatching periods, size at hatching, and first whorl diameter and is inversely related to the number of eggs per

capsule and the length of the precompetent period (Kohn, 2012), characteristics that still remain unknown for *C. ermineus*. It is worth mentioning that, if they were not fertilized, this could also affect their final size.

The pale orange eggs of *C. ermineus* were evident through the semi-transparent capsule walls. Coloured eggs occur in very few species of *Conus*, being pinky in *Conus abbreviatus* Reeve, 1843, *Conus lividus* Hwass, 1792, and *Conus stearnsii* (Conrad, 1869), and yellowish in *Conus jaspideus pygmaeus* Gmelin, 1791 and *Conus imperialis* Linnaeus, 1758 (Bandel, 1976; D’Asaro, 1986; Kohn, 1961b; Ostergaarg, 1950). In molluscs (and other invertebrates) egg colour seems to advertise potential predators on the presence of noxious compounds inside (Giglio et al., 2022). Toxins have been reported as being restricted to egg in *Conus victoriae* Reeve, 1843 (Safavi-Hemami et al., 2011). However, our observations do not allow us to provide any information on toxicity or biological implications in natural conditions for the coloured eggs of *C. ermineus*, as there has been a single oviposition event in artificial conditions.

Lifestyle of Dimorphilus oophagus sp. nov.

Egg capsules containing worms had damaged eggs and, in some instances, empty eggshells, while some of the worms had egg-like content in the anterior part of their digestive system, likely supporting they might be feeding upon the snail eggs. However, it must be kept in mind that this behaviour might be coincidental and further analyses are needed to determine whether it may occur also in natural conditions. Dinophilids most often feed on suspended food particles brought to their mouth by ciliary currents or by dislodging food from the substrate using their proboscis, with their diet usually including bacteria, protozoa, unicellular algae, diatoms, and detritus (Jennings & Gelder, 1969). To the best of our knowledge, there are no previous reports of oophagia, despite *Dinophilus vorticoides* Schmidt, 1848 has been found inside buoyant mollusc eggs (Worsaae et al., 2019). However, there are other oophagous polychaetes, including the hermit crab symbiotic spionids, *Polydora robi* Williams, 2000, and *Dipolydora commensalis* (Andrews, 1891), which feed on fertilized eggs and developing embryos of their host (Dauer, 1991; Williams, 2000) and the cephalopod symbiotic capitellids, *Capitella ovincola* Hartman, 1947 and *Capitella minima* (Langerhans, 1880). Despite there is no information on the influence on host hatching success for the spionids, the capitellids have been reported to improve the host hatch rate, so that their behaviours seem to be closer to a mutualism than to a parasitism (Martin & Britayev, 2018). The latter illustrates another polychaete-mollusc interaction.

Further studies are certainly required to assess the real significance of the oophagic behaviour in our new species, prior to qualify the relationship between *D. oophagus* sp. nov. and *C. emineus* as any type of symbiosis or, even as symbiotic. However, there are some evidences that may help. The egg capsules seemed

not to be damaged, and did not show openings, suggesting that the worms could enter the capsule during its formation. In addition, no worms were found in the tank water. Therefore, the worms could be present as resting or quiescent stages inside the specimens of *C. emineus* or *C. purpuracens*. In the first case worms' development could be triggered by hatching, while in the second case could be triggered by the matting parade, followed by a quick transfer to the egg capsules laid by the other species. Whether *D. oophagus* sp. nov. is a symbiotic (either a parasite, commensal, or mutualist), certainly requires further studies. However, quiescent states have never been reported in *Dimorphilus*. Alternatively, the worms could have been introduced to the tank together with the food or the coral-like rocks, which then infested the egg masses of *C. ermineus*. In this case, *D. oophagus* sp. nov. could not be a symbiont, but an egg feeder, either opportunistic or specialised. The last evidence derives from the fact that the specimens of *D. oophagus* sp. nov. kept isolated in captivity did not survive more than two days, which could be caused by a normal ending of a short life cycle (that seems to be typical in the species of the family) or by the fact that the specimens cannot survive outside the egg masses of its host. The meaning of these two possibilities is obviously different, as the first one did not imply a symbiotic relationship, while the second could certainly be related with a symbiotic behaviour. All these hypotheses are certainly intriguing and would require further analyses to be clarified.

Conclusions

This manuscript presents the first detailed description of the eggs (colour, shape, and size) of *C. ermineus*, a fish hunting cone snail from the Gulf of Mexico. Here we include the morphological description of the egg masses

and egg capsules deposited in captivity and compare them with those described in nature by other conspecifics. In addition to the morphological characterization of the cone snail eggs, we have identified and described a novel meiobenthic annelid species, *Dimorphilus oophagus* sp. nov. (Dinophilidae) that was found inside the egg capsules of the cone snail. The discovery of this new annelid worm is supported by molecular (COI) and morphological evidence. Behavioural observations allow us to discuss on alternative hypotheses to explain the lifestyle of the new worm species (i.e., symbiotic or not symbiotic), including the first observation of a dinophilid feeding upon “eggs” of a cone snail. Further studies are required to clarify the biological and ecological implications of the association between *D. oophagus* sp. nov. and *C. emineus*.

Acknowledgments

This work was funded by NIH (grant# ROIGM144719). We would like to thank Sean Christensen for the provision of the eggs of *Conus ermineus* and Dr. Elaine Seaver for her help in the initial identification of the new annelid and for her comments on the initial version of the manuscript. We would also like to thank Dr. Samuel Espino for the images of *C. ermineus*. Finally, we would also like to thank the reviewers for their valuable comments that helped to improve the original manuscript. Matías L. Giglio and Paula Flórez Salcedo contributed equally to this article.

References

Bandel, K. (1976). Spawning, development and ecology of some higher Neogastropoda from the Caribbean Sea of Colombia (South America). *Veliger*, 19, 176–193.

- Cruz, L.J. & Olivera, B.M. (1978). Mating, spawning, development and feeding habits of *Conus geographus* in captivity. *Nautilus*, 92, 150–153.
- D'Asaro, C. (1970a). Egg capsules of prosobranch mollusks from South Florida and the Bahamas and notes on spawning in the laboratory. *Bull. Mar. Sci.*, 20, 414–440.
- D'Asaro, C. (1970b). Egg capsules of some prosobranchs from the Pacific coast of Panama. *Veliger*, 13, 37–43.
- D'Asaro, C. (1986). Egg capsules of eleven marine prosobranchs from northwest Florida. *Bull. Mar. Sci.*, 39, 76–91.
- Dauer, D.M. 1991. Functional morphology and feeding behavior of *Polydora commensalis* (Polychaeta: Spionidae). *Ophelia Suppl.*, 5, 607–614.
- Duda, T.F. & Palumbi, S.R. (2004). Gene expression and feeding ecology: Evolution of piscivory in the venomous gastropod genus *Conus*. *Proc. Biol. Sci.*, 271, 1165–1174.
- Giglio, M.L., Boland, W. & Heras, H. (2022). Egg toxic compounds in the animal kingdom. A comprehensive review. *Nat. Prod. Rep.*, 39, 1938–1969.
- Gonçalves, P. B., Lima, S. F. B., Oliveira, G. S. P. & Lucena, R. A. (2017). On the oviposition and egg masses of *Conus regius* (Neogastropoda: Conidae) from northeastern Brazil. *Biota Neotropica.*, 17, e20170434.
- Jennings, J.B., & Gelder, S.R. (1969). Feeding and digestion in *Dinophilus gyrocolliatus* (Annelida: Archiannelida). *J. Zool.*, 158, 441–451.
- Jones, E.R. & Ferguson, F.F. (1957). *Dinophilus* (Archiannelida) in the United States. *Amer. Midland Natur.*, 57, 440–449.
- Kalyanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler, A., and Jermin, L.S. (2017). ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat. Methods*. 14, 587–589.
- Katoh, K., Rozewicki, J., and Yamada, K.D. (2017). MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief. Bioinform.*, 20, 1160–1166.

- Kohn, A.J. (1961a). Studies on spawning behavior, egg masses, and larval development in the gastropod genus *Conus*, Part I. Observations on nine species in Hawaii. *Pacific Sci.*, 15, 163–180.
- Kohn, A.J. (1961b). Studies on spawning behavior, egg masses and larval development in the gastropod genus *Conus*, Part II. Observations in the Indian Ocean during the Yale Seychelles Expedition. *Bull. Bingham Ocean. Lab.*, 17, 3–51.
- Kohn, A.J. (2012). Egg size, life history, and tropical marine gastropod biogeography. *Amer. Malacol. Bull.*, 30, 163–174.
- Lebour, M.V. (1945). The eggs and larvae of some prosobranchs from Bermuda. *J. Zool.*, 114, 462–489.
- Letunic, I. & Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.*, 49, W293–W296.
- Lobo Arteaga, J., Teixeira, M., Borges, L., Ferreira, M., Hollatz, C., Gomes, P., Sousa, R., Ravara, A., Costam, M.H. & Costa, F.O. (2016). Starting a DNA barcode reference library for shallow water polychaetes from the southern European Atlantic coast. *Mol. Ecol. Resour.*, 16, 298–313.
- Martin, D. & Britayev, T.A. (2018). Symbiotic polychaetes revisited: An update of the known species and relationships (1998–2017). *Ocean. Mar. Biol.*, 56, 371–448.
- Martín-Durán, J.M., Vellutini, B.C., Marlétaz, F., Cetrangolo, V., Cveticic, N., Thiel, D., Henriët, S., Grau-Bové, X., Carrillo-Baltodano, A.M., Gu, W., Kerbl, A., Marquez, Y., Bekkouche, N., Chourrout, D., Gómez-Skarmeta, J.L., Irimia, M., Lenhard, B., Worsaae, K. & Hejnol, A. (2021). Conservative route to genome compaction in a miniature annelid. *Nat. Ecol. Evol.*, 5, 231–242.
- Minh, B.Q., Nguyen, M.A.T. & von Haeseler, A. (2013). Ultrafast Approximation for Phylogenetic Bootstrap. *Mol. Biol. Evol.*, 30, 1188–1195.
- Monnier, E., Limpalaër, L., Robin, A. & Roux, C. (2018) *A taxonomic Iconography of Living Conidae*, Vol 2. ConchBooks. 602 pp.
- Natarajan, A.V. (1957). Studies on the egg masses and larval development of some prosobranchs from the Gulf of Mannar and the Palk Bay. *Indian Academy of Science*, 46, 170–228.
- Nguyen, L.T., Schmidt, H.A., von Haeseler, A. & Minh, B.Q. (2015). IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.*, 32, 268–274.
- Nybakken, J. (1967). Notes on the egg capsules and larval development of *Conus purpurascens* Broderip. *Veliger*, 12, 480–481.
- Olivera, B.M., Corneli, P.S., Watkins, M. & Fedosov, A. (2014). Biodiversity of cone snails and other venomous marine gastropods: Evolutionary success through neuropharmacology. *Annu. Rev. Anim. Biosci.*, 2, 487–513.
- Olivera, B.M., Fedosov, A., Imperial, J.S. & Kantor, Y. (2017). Physiology of envenomation by conoidean gastropods. In *Physiology of Molluscs: A Collection of Selected Reviews* (Vol. 1, Issue May).
- Ostergaard, J. (1950). Spawning and Development of Some Hawaiian Marine Gastropods. *Pacific Sci.*, 4, 75–115.
- Penchaszadeh, P. (1984). Observations on the spawn of three species of *Conus* from the Golfo Triste, Venezuela. *Veliger*, 27(1), 14–18.
- Puillandre, N., Bouchet, P., Duda, T. F., Kauferstein, S., Kohn, A. J., Olivera, B. M., Watkins, M., & Meyer, C. (2014). Molecular phylogeny and evolution of the cone snails (Gastropoda, Conoidea). *Mol. Phyl. Evol.*, 78, 290–303.
- Ramiro, I.B.L., Bjørn-Yoshimoto, W.E., Imperial, J.S., Gajewiak, J., Salcedo, P.F., Watkins, M., Taylor, D., Resager, W., Ueberheide, B., Bräuner-Osborne, H., Whitby, F.G., Hill, C.P., Martin, L.F., Patwardhan, A., Concepcion, G.P., Olivera, B.M. & Safavi-Hemami, H. (2022). Somatostatin venom analogs evolved by fish-hunting cone snails: From prey capture behavior to identifying drug leads. *Sci. Adv.*, 8, eabk1410.

- Safavi-Hemami, H., Siero, W.A., Kuang, Z., Williamson, N.A., Karas, J.A., Page, L.R., MacMillan, D., Callaghan, B., Kompella, S.N., Adams, D.J., Norton, R.S. & Purcell, A.W. (2011). Embryonic toxin expression in the cone snail *Conus victoriae*: Primed to kill or divergent function? *J. Biol. Chem.*, 286, 22546–22557.
- Schmidt, O. (1857). Zur Kenntnis der Turbellaria, Rhabdocoela und einiger anderer Wuermer des Mittelmeeres. *Sitzungsberichte der Kaiserliche Akademie der Wissenschaften, Wien, Mathematisch-Naturwissenschaftliche Klasse.*, 23, 347–366.
- Terlau, H. & Olivera, B.M. (2004). Conus venoms: A rich source of novel ion channel-targeted peptides. *Physiol. Rev.*, 84(1), 41–68.
- Williams, J.D. (2000). A new species of *Polydora* (Polychaeta: Spionidae) from the Indo-West Pacific and first record of host hermit crab egg predation by a commensal polydorid worm. *Zool. J. Linn. Soc.*, 129, 537–548.
- WoRMS Editorial Board. (2022). *World Register of Marine Species (WoRMS)*. <https://www.marinespecies.org>.
- Worsaae, K., Kerbl, A., Di Domenico, M., Gonzalez, B.C., Bekkouche, N. & Martínez, A. (2021). Interstitial annelida. *Diversity.*, 13, 77.
- Worsaae, K., Kerbl, A., Vang, Á., & Gonzalez, B. C. (2019). Broad North Atlantic distribution of a meiobenthic annelid – against all odds. *Scientific Reports*, 9(1), 1–13. <https://doi.org/10.1038/s41598-019-51765-x>.
- Zehra, I., & Perveen, R. (1991). Egg capsule structure and larval development of *Conus biliosus* (Röding, 1798) and *C. Coronatus* Gmelin, 1791, from Pakistan. *Journal of Molluscan Studies*, 57(2), 239–248.