Title

A Buoyancy-Controlled Lagrangian Camera Platform for In Situ Imaging of Marine Organisms in Midwater Scattering Layers

Journal

IEEE Journal of Oceanic Engineering, 43(3)

ISSN

0364-9059

Authors

Berkenpas, Eric J
Henning, Bradley S
Shepard, Charles M
et al.

Publication Date

2018-07-01

DOI

10.1109/joe.2017.2736138

Journal of
Molluscan Studies

The Malacological Society of London

Journal of Molluscan Studies (2017) **83**: 194–199. doi:10.1093/mollus/eyx008
Advance Access publication date: 3 March 2017

Morphological description of egg masses and hatchlings of *Lolliguncula diomedeae* (Cephalopoda: Loliginidae)

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(Received 24 November 2016; editorial decision 1 February 2017)

ABSTRACT

We describe the morphology of egg masses and hatchlings of the squid, *Lolliguncula diomedeae* (Hoyle, 1904) based on individuals hatched in the laboratory from two egg masses collected in the Gulf of California near Santa Rosalía, Baja California Sur, Mexico, and identified with molecular techniques. The characteristics of the egg mass are described and compared with other related species. Gross morphology, chromatophore pattern and the arrangement of arm and tentacle suckers of hatchlings are described. Among squids in the family Loliginidae, hatchlings of this species represent the second smallest described to date. The morphology of egg masses and paralarvae of related and sympatric species are compared, providing a tool for identification that may foster the understanding of the population dynamics and life history of this species.

INTRODUCTION

The family Loliginidae comprises 10 genera and 47 species (Jereb, Vecchione & Roper, 2010), all of which inhabit coastal areas and continental shelves. Loliginid hatchlings are planktonic and range in size from 1.1 to 10 mm dorsal mantle length (DML) (Segawa, 1990; Nabhitabhata *et al.*, 2005), with a mean of 3.0 mm (standard deviation (SD) = 1.7 mm) DML for 16 studied species (Villanueva *et al.*, 2016). In loliginid squid, the appearance of schooling behaviour is considered the end of the planktonic phase. The duration of this planktonic phase seems to be affected by hatchling size, but data are available from only a few species (Villanueva *et al.*, 2016). Thus, the size of loliginid hatchlings may have important implications for their life history and survival during the critical paralarval phase. Therefore, a better understanding of paralarval biology is crucial to further our knowledge of cephalopod population dynamics, life cycles and fisheries (Boyle & Rodhouse, 2005).

The genus *Lolliguncula* Steenstrup, 1881 is composed of four species of small coastal squids (Jereb *et al.*, 2010). Three of these cohabit in the eastern Pacific and one is present in the western Atlantic. *Lolliguncula diomedeae* (Hoyle, 1904) ranges from Mexico to Peru and overlaps with the ranges of *Lolliguncula panamensis* Berry, 1911b and *Lolliguncula argus* Brakoniecki & Roper, 1985. Little is known of the biomass of any of these species, but *L. diomedeae* and *L. panamensis* together represent 2–4% of the bycatch weight taken in the shrimp trawl fisheries of southwest Mexico (Alejo-Plata, Cerdenares & Herrera-Galindo, 2001), which suggests that these squids are quite abundant. Nonetheless, nearly every aspect of the life history of *L. diomedeae* remains unknown (Alejo-Plata, Gómez-Márquez & Herrera-Galindo, 2015). This paper provides the first description of the morphology of the egg mass and paralarvae of *L. diomedeae*, based on egg masses collected from the wild and identified using molecular techniques.

MATERIAL AND METHODS

Animals

Egg masses were collected by shark fishermen between 5–7 August 2015 in the Gulf of California near Santa Ana (27.6625°N, 112.5997°W), about 40 km northwest of Santa Rosalía, Baja California Sur, Mexico (Fig. 1). The egg masses were found on gillnets that had been deployed 750–1000 m from the shore, over rocky reef habitat in water *c.* 70 m deep. Both egg masses were affixed to the gillnet floatlines *c.* 20 m above the seafloor. Hydrographic properties of the water column (27.6703°N, 112.4911°W) were assessed on 15 August 2015 with a conventional profiler (SBE19plusV2, Sea Bird Electronics, Redmond, WA, USA). Surface temperature was 28.2 °C and at 50 m depth the value was 23.3 °C, with essentially no surface mixed-layer. Salinity at 50 m depth was 35.01‰ and oxygen was 126 µmol/kg. A chlorophyll *a* peak was observed between 8 and 48 m, with a maximum value of 1.2 mg/m^3 at 22 m depth. These conditions were likely similar to those at the time of egg deposition.

The egg masses were kept in minimally insulated plastic containers for 12–24 h before they could be transferred to a tank in the laboratory in Santa Rosalía for incubation. Embryos from the first egg mass had died by 6 August 2015, and a capsule from this mass was immediately preserved in 95% ethanol. The second egg mass, collected on 7 August 2015, contained live embryos and was transferred to a 1-l container with three large perforations (6 × 10 cm)

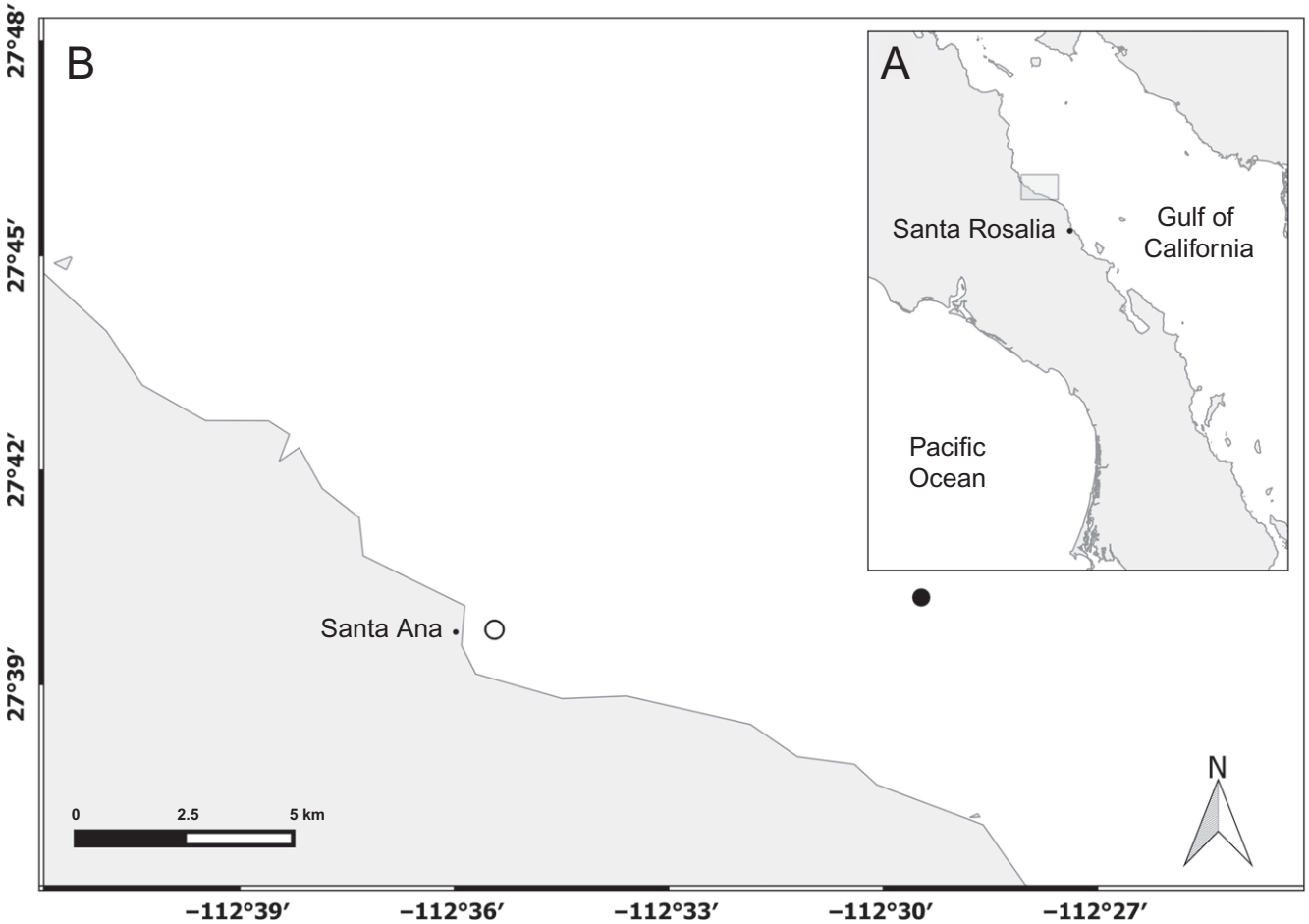

Figure 1. A. Location of sample collection in the Gulf of California. **B.** Enlargement of sampling location, showing point of collection of egg masses (white circle) and point where hydrographic data were obtained (black circle).

covered by mesh to facilitate aeration. This container was then placed in a 890-l circular tank (1.22 m diameter × 0.76 m depth; RT-430 INS. Frigid Units, Toledo, OH) containing aerated seawater at 15 °C with a salinity of 34‰. The holding tank was a closed system with two recirculating loops. One loop provided chilling (AE62B, Pentair Aquatic Eco-Systems, Apopka, FL) and filtering through cartridge filters (50 and 5 µm; VF25, Pentair) and a carbon canister (FCB50H, Pentair). A second loop provided biological filtration (Trickle 300, Pentair).

DNA extraction, PCR amplification, sequencing and sequence analysis

Total genomic DNA was extracted from four ethanol-fixed embryos of each egg mass (n = 8), using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). The manufacturers protocol was modified to maximize the amount of DNA extracted from the embryos (*c.* 100 µg each), by (1) pulverizing the entire embryo using pestles to facilitate cell lysis, (2) halving the reagent volumes (with the exception of the washes), (3) extending the final room-temperature incubation to 10 min and (4) eluting DNA twice instead of once (first in 100 µl, then in 50 µl of elution buffer AE).

A portion of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) was amplified using the primer pair LCO1490 and HCO2198 (Folmer *et al.*, 1994). PCR amplifications were performed in 100 µl volumes with 10 µl of 10× NH_4 reaction buffer (Bioline), 4 µl of $MgCl_2$ (50 mM), 2 µl of dNTPs (10 mM), 1 µl each of the primers (25 µM), 1 µl of Taq DNA polymerase (5 units/µl, Bioline), 1 µl of template DNA and 80 µl of deionized water. The PCR protocol consisted of an initial denaturation for 7 min at 94 °C; followed by 39 cycles of denaturation for 10 s at 94 °C, annealing for 30 s at 46 °C, and extension for 60 s at 72 °C; and a final extension at 72 °C for 7 min. The PCR products were stained with a fluorescent dye (EZ-Vision Three, Amresco Inc.) and visualized on a 0.8% agarose gel. Sanger sequencing was used to obtain both forward and reverse sequences from eight specimens (ABI 3730xl sequencer, Elim Biopharmaceuticals Inc., CA).

Consensus COI sequences from each of the eight samples were aligned using Multiple Sequence Comparison by Log-Expectation, checked for sequencing errors and trimmed to a length of 658 bp within Geneious Pro v. 7.1.4. Unique haplotypes were identified using COLLAPSE v. 1.2 (Posada, 2004) and uploaded to GenBank with the accession numbers KX610666–KX610667. Unique haplotypes were then compared with GenBank sequences using BLAST (Altschul *et al.*, 1990). Sequences with the highest similarity to our unique haplotypes were downloaded and uncorrected p-distances between our unique haplotypes and highly similar GenBank sequences were calculated using MEGA v.7 (Kumar, Stecher & Tamura, 2016) to determine the identity of our samples.

Observations of egg masses and live paralarvae

Each egg mass was photographed in water to document its size and structure. Observations and measurements of the egg mass and egg capsules were made on the basis of these photographs. Hatching occurred after 6 d. Ten paralarvae were transferred to Petri dishes, anaesthetized by adding a few drops of 70% ethanol

and photographed under a binocular stereomicroscope. Prior to fixation in 4% buffered formalin in seawater, individuals were euthanized by an overdose of the anaesthetic.

Morphological descriptions of the paralarvae were based on photographs of these ten anaesthetized live specimens. Nine parameters for morphological measurement were identified based on those from Roper & Voss (1983): DML, ventral mantle length, total length, head length, head width, eye diameter, funnel length, fin length and fin width. All measurements were made using the software LAS CORE v. 4.1 (Leica Microsystems, Wetzlar, Germany). After obtaining these measurements, the ratio between each parameter and DML, referred to as an index, was calculated following the methods of Roper & Voss (1983). Measures are expressed as mean ± SD throughout the text.

The chromatophore patterns of the dorsal, ventral and lateral surfaces were documented by creating a schematic drawing of each paralarva. Chromatophores were codified as *d* (dark red) or *y* (yellow) and were assigned to transverse rows along the anteroposterior axis of each surface as described by Fernández-Álvarez *et al.* (2016). For example, the dorsal side of the mantle might have the pattern *d*1*y*1 + *y*4 + *y*2 + *y*1, meaning one dark and one yellow chromatophore in the anterior margin of the mantle, four yellow chromatophores in the second row, two yellow chromatophores in the third row and one yellow chromatophore near the posterior tip of the mantle. The resulting schematics were based on the most representative patterning observed from all individuals.

Observations under scanning electron microscopy

Fixed specimens were washed in filtered seawater followed by dehydration in increasing concentrations of ethanol (20, 30, 40, 50, 70, 80, 90 and 96%) until they were saturated in absolute ethanol. Each ethanol bath lasted 10 min. After complete dehydration in the ethanol series, the samples were dried to critical point in a Bal-Tec CPD 030 Drier using CO_2 as the transition liquid. Samples were then mounted on stubs with double-sided conductive sticky-tape to orientate them in the preferred position and sputter coated with gold–palladium in a Quorum Q150RS. Finally, the samples were examined using a scanning electron microscope Hitachi S-3500 N with a working voltage of 5 kV at the Institut de Ciències del Mar (ICM-CSC) in Barcelona. The general morphology and the number and arrangement of the suckers of arms and tentacles were studied.

RESULTS

Molecular identification of the embryos and paralarvae

We identified two unique haplotypes, one from each egg mass sampled. These haplotypes were both 99% similar, with 91% coverage, to GenBank sequence EU735357. That sequence was derived from a morphologically identified specimen of *Lolliguncula diomedeae* from the tropical Pacific coast of Mexico (Lindgren, 2010; Sales *et al.*, 2014). The uncorrected p-distances among the three haplotypes of *L. diomedeae* were 0.2–0.7%, which is similar to intraspecific distances in other species of the genus (Sales *et al.*, 2014). Thus, both egg masses contained embryos of *L. diomedeae*. It should be noted that our molecular identification relies on the correct morphological identification of the specimen from which the sequence EU735357 was obtained.

Egg-mass structure

Examination of fresh, live egg masses revealed a typical loliginid egg mass structure (Boletzky, 1998). Each egg mass consisted of *c.* 20–30 soft, translucent egg capsules (Fig. 2A). Individual capsules were 8–9 mm wide with a maximum length of 85 mm and they did not have an attachment region devoid of eggs as occurs in related species (see below). Each capsule contained up to 70 eggs (range 52–70, $n = 3$), which were arranged helicoidally in batches of four eggs each measuring 4.14 ± 0.25 mm in diameter ($n = 10$) (Fig. 2B).

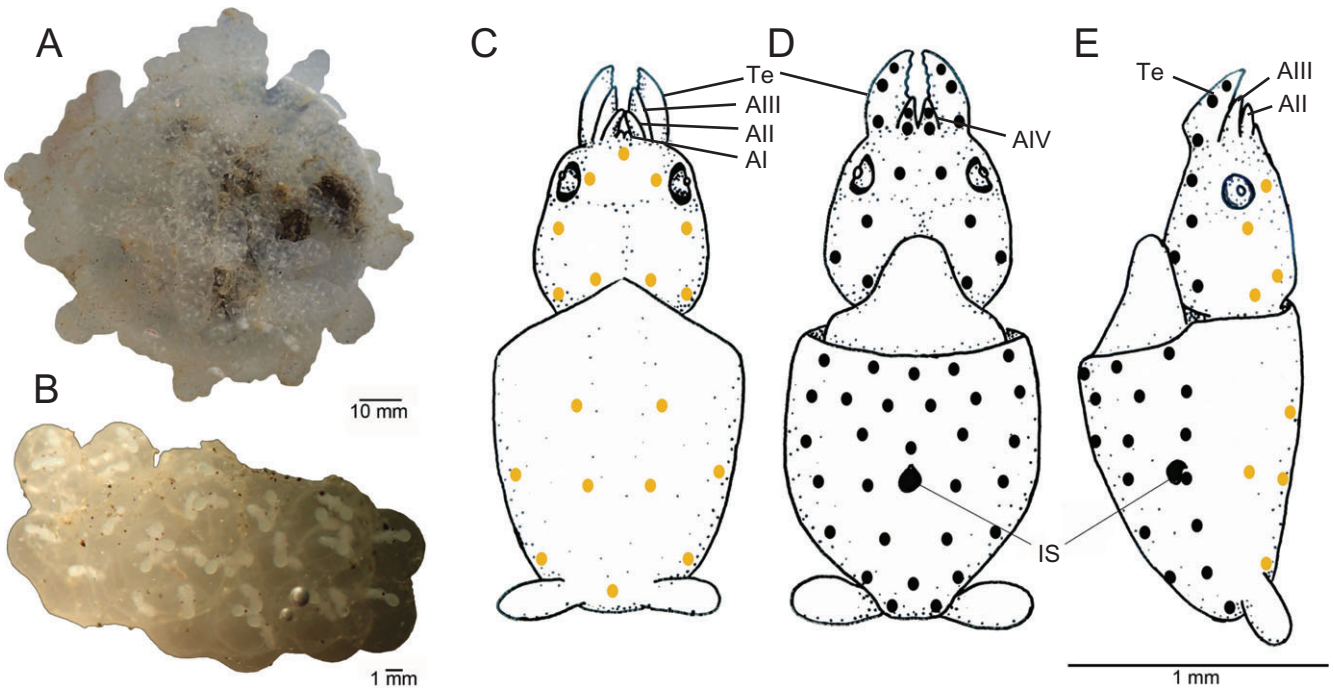

Figure 2. *Lolliguncula diomedeae.* **A.** Live egg mass. **B.** Part of an egg capsule, showing arrangement of eggs. **C–E.** Schematic drawings of external morphology of hatchling, showing chromatophore pattern. **C.** Dorsal view. **D.** Ventral view. **E.** Lateral view. Abbreviations: AI, arm I; AII, arm II; AIII, arm III; AIV, arm IV; IS, ink sac; Te, tentacle.

Gross morphology of hatchlings

Hatchling paralarvae in the fresh state (1.28 ± 0.05 mm DML, n = 10) possessed a typical loliginid morphology (Fig. 2C–E). The head was narrower than the mantle and bore the primordia of arm pairs I–IV and the tentacles. Fins were relatively well developed, elliptical and separated at the subterminal part of the mantle. Morphometric data and index ratios are summarized in Table 1.

A detailed examination of arms and tentacles was carried out with SEM. Tentacles were larger than the largest arms, with arm pair III being the longest arms (Fig. 3), arms pairs II and IV being about the same length (Fig. 3B, C) and arms I existing just as a small papillae between arm pair II (Fig. 3A). Arm pair I was devoid of suckers, arm pairs II and IV had a single sucker on each arm and arm pair III had a row with two suckers (Fig. 3D). Each tentacle had three rows of two suckers (Fig. 3D).

Chromatophore pattern

Chromatophores on the dorsal surface were yellow (Fig. 2C), whereas ventral chromatophores were dark red, almost black when contracted (Fig. 2D). The dorsal surface of the head exhibited nine chromatophores (although one specimen with six and another with 11 were documented) that were arranged in five rows with the pattern $y1 + y2 + y2 + y2 + y2$. The ventral surface of the head had eight chromatophores ($d2 + d2 + d2 + d2$), but a single asymmetrical chromatophore between the second and third ventral-head rows was observed in one specimen. Posterior to each eye, three ventral chromatophores formed a triangular patch (Fig. 2D), referred to as the 'cheek patch' in some loliginids (Vecchione & Lipinski, 1995).

Arm pairs I and III were devoid of chromatophores in all individuals, although one yellow chromatophore was present on one arm of pair II of a single specimen. All individuals had a basal dark chromatophore on arm pair IV, however six individuals possessed an additional chromatophore that was either yellow or dark on this arm pair. All individuals had three dark chromatophores on the tentacles and four individuals had an additional yellow or dark chromatophore.

The mantle featured nine to 12 dorsal yellow chromatophores arranged in four transverse rows and 26–30 ventral dark chromatophores arranged in six to seven rows. Dorsal chromatophores were usually distributed in the pattern $y2 + y4 + y2 + y1$ (Fig. 2C); the ventral pattern tended to be $d5 + d6 + d5 + d4 + d4 + d3 + d2$ (Fig. 2D).

DISCUSSION

Egg masses of Lolliguncula *species*

Egg masses of *Lolliguncula diomedeae* match general features of loliginid egg masses (Boletzky, 1998): elongate and gelatinous capsules attached to the substratum by a fixating jelly. To the best of our knowledge, only two other descriptions of *Lolliguncula* egg masses are available. Hall (1970) reported wild egg masses of *Lolliguncula brevis* Blainville, 1823 from Tampa (Florida, USA) to consist of individual clavate capsules 100–130 mm long, directly attached to the substratum. The distal, bulbous end of the capsules contained 56–88 eggs, whereas the proximal end was devoid of eggs. Hereafter, *L. brevis* collected from the Gulf of Mexico off Tampa will be referred to as 'northern *L. brevis*'. In contrast, Zaleski, Perez & Gandara Martins (2012) reported a different egg-mass morphology for this species from the southwest Atlantic (Brazil). In this case, the egg masses consisted of clutches of 18–73 globule-shaped capsules 94–285 mm long and 60–70 mm wide with 1–38 eggs inside. Each capsule had a peduncle (<50% of capsule length) that was fused with the peduncles of other capsules to a common stalk affixed to the substrate. As Zaleski *et al.* (2012) pointed out, the differences in egg mass morphologies together with other morphological differences suggest that northern and southern *L. brevis* populations may be distinct species. This conclusion is supported by the significant genetic distance between northern and southern *L. brevis* (Sales *et al.*, 2014).

Egg masses of *L. diomedeae* as described in this report are quite different from either of the above descriptions. They are shorter and, more importantly, lack an attachment region devoid of eggs, a feature common to both forms of *L. brevis*. Unlike northern *L. brevis*, *L. diomedeae* egg masses have a single point of attachment to the substrate and are composed of capsules with filiform rather than clavate morphology. Egg capsules of *L. diomedeae* differ from southern *L. brevis* in their shape and width, which are globular and relatively wider in southern *L. brevis* compared with *L. diomedeae*.

Chromatophores of Lolliguncula *species*

Significant differences in the chromatophore distribution exist between paralarvae of northern *L. brevis* (McConathy, Hanlon & Hixon, 1980) and *L. diomedeae*. At hatching, northern *L. brevis* paralarvae (1.8 mm DML) are larger than those of *L. diomedeae* as reported here (1.28 mm DML). The total number of chromatophores (93) in *L. brevis* is higher than the number in *L. diomedeae* (53) and further differences arise in their arrangement and coloration. On the tentacles, northern *L. brevis* has three yellow and three dark red chromatophores, whereas *L. diomedeae* hatchlings usually have only three dark red chromatophores. On the dorsal surface of the mantle, northern *L. brevis* has three chromatophores arranged in two rows ($y2 + y1$), whereas *L. diomedeae* has nine to 12 yellow chromatophores arranged in four transverse rows.

Although chromatophore distribution differs between *L. diomediae* and northern *L. brevis*, some similarities do exist between these species. Chromatophores on the dorsal surface of the head share a common spatial arrangement in both species and are equal in number. Both species also have chromatophores arranged in seven rows on the ventral mantle surface, although *L. brevis* has 39 chromatophores

Table 1. Measurements (mm) of morphometric parameters and ratios (%) of each parameter in relation to dorsal mantle length of anaesthetized paralarvae of *Lolliguncula diomedeae* (n = 10).

	DML	VML	TL	MW	HL	HW	ED	FuL	FL	FW
Average	1.28	1.02	2.24	1.01	0.67	0.65	0.25	0.53	0.17	0.84
SD	0.05	0.08	0.17	0.06	0.04	0.03	0.02	0.07	0.03	0.05
Range	1.20–1.39	0.86–1.13	1.99–2.65	0.88–1.10	0.61–0.77	0.62–0.72	0.22–0.27	0.44–0.64	0.12–0.22	0.76–0.91
		VMLI	TLI	MWI	HLI	HWI	EDI	FuLI	FLI	FWI
Average		78	175	79	52	50	19	42	14	66
SD		5	14	7	4	2	1	5	2	3
Range		69–85	147–200	63–87	44–58	47–54	17–20	35–50	10–17	61–70

Abbreviations: DML, dorsal mantle length; VML, ventral mantle length; TL, total length; MW, mantle width; HL, head length; HW, head width; ED, eye diameter; FuL, funnel length; FL, fin length; FW, fin width; VMLI, ventral mantle length index; TLI, total length index; MWI, mantle width index; HLI, head length index; HWI, head width index; EDI, eye diameter index; FuLI, funnel length index; FLI, fin length index; FWI, fin width index.

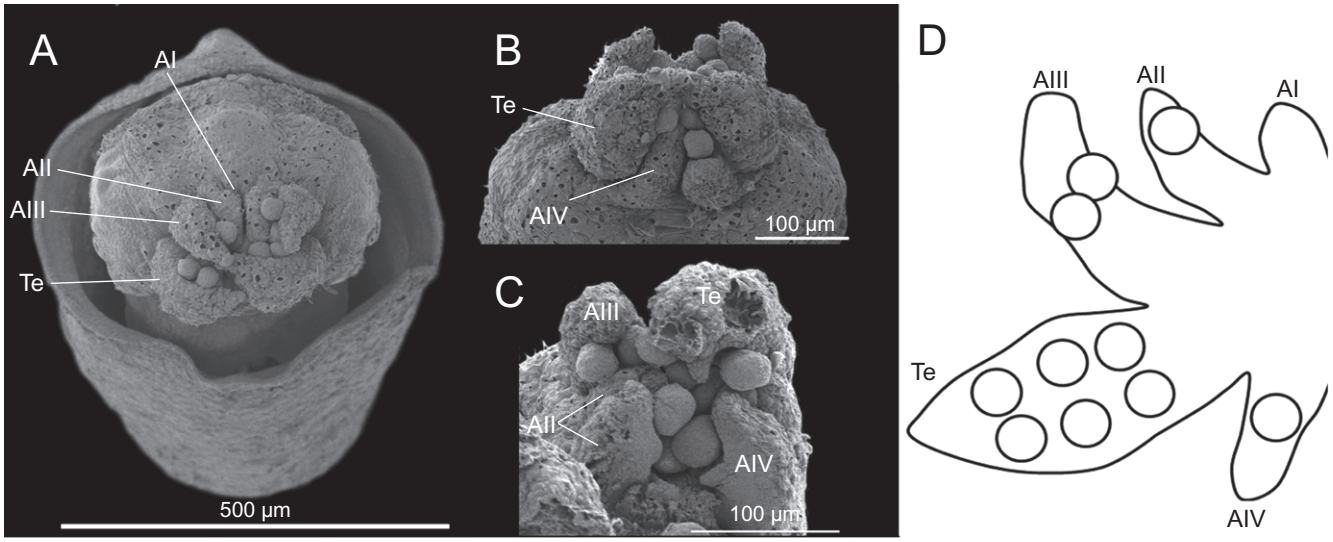

Figure 3. *Lolliguncula diomedeae.* **A.** SEM image of frontal view of hatchling; arms I–III and tentacles are visible. **B.** Anteroventral view of head; arms IV and tentacles are visible. **C.** Arm crown of a sagittally sectioned paralarvae; arm pair I is hidden by arms II. **D.** Schematic drawing of oral view of hatchling arm crown, showing relative length of arms, tentacles and number and position of suckers. Abbreviations: AI, arm I; AII, arm II; AIII, arm III; AIV, arm IV; Te, tentacle.

Table 2. Differences between the sympatric loliginid hatchlings of *Lolliguncula diomedeae*, *Doryteuthis gahi* and *D. opalescens*.

	DML (mm)	Number of chromatophores in 'cheek patch'	Dorsal mantle chromatophores	Ventral mantle chromatophores
L. diomedeae	1.20–1.39	3	Only yellow	Only dark red
D. gahi	3.1–3.4	4	Yellow and dark	Yellow and dark
D. opalescens	2.5–3.2	3	Yellow and dark	Yellow and dark

Abbreviation: DML, dorsal mantle length.

and *L. diomedeae* appears to have no more than 30. Finally, the triangle of three ventral chromatophores found posterior to the eyes in the 'cheek patch' (*sensu* Vecchione & Lipinski, 1995) is a characteristic shared between both species.

Chromatophores of sympatric loliginids in the eastern Pacific

The differences in the spatial distribution and pigmentation of chromatophores of loliginid paralarvae are useful morphological features to distinguish species that are commonly encountered together in plankton tows. This is particularly true for congeneric species. For example, McConathy *et al.* (1980) found that the chromatophore pattern was effective for distinguishing between three loliginid species that inhabit the northwestern Atlantic: the northern *L. brevis*, *Doryteuthis plei* and *Doryteuthis pealeii*. Vecchione (1982) later identified wild *L. brevis* paralarvae based on their chromatophore pattern and provided morphological descriptions of this species in posthatchling stages.

Lolliguncula diomedeae coexists with several other loliginid species in the eastern Pacific and mixed captures may occur in plankton tows. The northernmost range limit of *Doryteuthis gahi* in the Southern Hemisphere partially overlaps with the southern portion of the range of *L. diomedeae* (Jereb *et al.*, 2010). However, the hatchlings of both species are quite different: *D. gahi* is a species adapted to cold-water environments (Arkhipkin, Laptikhovsky & Middleton, 2000) and produces large (3.1–3.4 mm DML) hatchlings. *Doryteuthis gahi* has also a different chromatophore pattern on the ventral surface of the head (Baron, 2003), with four dark chromatophores in the 'cheek patches' in comparison with the three in *L. diomedeae* (Fig. 2D). More significantly, *D. gahi* has dark chromatophores on the dorsal mantle and head and yellow ones on the ventral surface, while *L. diomedeae* has only yellow chromatophores in the dorsal surface and dark chromatophores in the ventral mantle.

Doryteuthis opalescens, a northern hemisphere species, also occurs with *L. diomedeae* at the southern limit of its range (Jereb *et al.*, 2010). Hatchlings of *D. opalescens* are again larger (2.7 mm DML; McConathy *et al.*, 1980) than those of *L. diomedeae* and have a different arrangement of chromatophores: the ventral mantle chromatophores are irregularly distributed and there are both yellow and dark chromatophores on the dorsal and ventral surfaces. However, the three ventral-head chromatophores of the 'cheek patches' are arranged in the same manner in both species.

Thus, hatchlings of both species of *Doryteuthis* that are known to overlap in distribution with *L. diomedeae* can now be reliably differentiated based on examination of size and chromatophore number, colour and orientation. The differences between the three species are summarized in Table 2. However, it should be noted that there are three other sympatric loliginids whose paralarvae remain undescribed and thus cannot be reliably identified from plankton tows: the congeneric *Lolliguncula panamensis* and *Lolliguncula argus*, and *Pickfordiateuthis vossi*.

ACKNOWLEDGEMENTS

We thank Castulo, Timothy H. Frawley, Patrick C. Daniel and El Programa Pesca Pelágica de Santa Rosalía for their valuable help in the collection of the egg masses and data. Thanks to José Manuel Fortuño (Electron Microscopy Service, ICM-CSIC) for his dedication and kindness during the SEM sessions and Federico Quattrocchi for his help with Figure 1. We thank the Associate Editor Janet Voight, Michael Vecchione and another anonymous reviewer for

their helpful comments on the manuscript. This study was funded by the research project CALOCEAN-2 (AGL2012-39077) from the Ministry of Economy and Competitiveness (MINECO) of Spain. F.A.F-A. was supported by a predoctoral fellowship (BES-2013-063551) and short stage grant during the summer of 2015 (EEBB-I-15-09631) from MINECO. E.P. was supported in part by the US National Science Foundation Graduate Research Fellowship Program under Grant No. DGE-114747. R.V. was funded by the Spanish Ministry of Education and Culture (Grant No. PRX15/00100). W.F.G. was funded by grants OCE 0850839, OCE 1338973-RAPID and IOS 142093-EAGER from the US National Science Foundation and N000140911054 from US Office of Naval Research.

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