Int. J. Dev. Biol. 65: 323-332 (2021)
https://doi.org/10.1387/ijdb.200157fo

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

The "Mexican dancer" in Ecuador: molecular confirmation, embryology and planktotrophy in the sea slug *Elysia diomedea*

LOLA MOREANO-ARROBO[1,#], OSCAR D. PÉREZ[1,2,3,#], FEDERICO D. BROWN[2,3], FERNANDA X. OYARZÚN*[,4,5,6], and CRISTIAN B. CANALES-AGUIRRE[6,7]

[1]Laboratorio de Biología del Desarrollo 113, Escuela de Ciencias Biológicas, Pontificia Universidad Católica del Ecuador, Ecuador, [2]Departamento de Zoología. Instituto de Biociências, Universidade de Sao Paulo, Brazil, [3]Centro de Biologia Marinha (CEBIMAR), Universidade de São Paulo, Brazil, [4]Centro de Investigación en Biodiversidad y Ambientes Sustentables (CIBAS), Universidad Católica de la Santísima Concepción, Concepción, Chile, [5]Departamento de Ecología, Universidad Católica de la Santísima de Concepción, Concepción, Chile, [6]Centro i~mar, Universidad de Los Lagos, Puerto Montt, Chile and [7]Núcleo Milenio de Salmónidos Invasores (INVASAL), Concepción, Chile

ABSTRACT ***Elysia diomedea* , otherwise known as the "Mexican dancer", aries in adult size and color across its geographical distribution in Ecuador. Because of morphological variation and the absence of genetic information for this species in Ecuador, we analyzed mtDNA sequences in three populations (Ballenita, La Cabuya, and Mompiche) and confirmed that individuals from the three locations belonged to *E. diomedea* and that there was no population structure that could explain their morphological differences. Next, we analyzed general aspects about the reproductive biology and embryology of this species. Live slugs from the Ballenita population were maintained and reproduced *ex situ*. Egg ribbons and embryos were fixed and observed by brightfield and confocal microscopy. We observed a single embryo per capsule, 98 embryos per mm² of egg ribbon, and compared the cleavage pattern of this species to that of other heterobranchs and spiralians. *E. diomedea* early development was characterized by a slight unequal first cleavage, occurrence of a 3-cell stage in the second cleavage, and the formation of an enlarged second quartet of micromeres. We observed clear yolk bodies in the egg capsules of some eggs ribbons at early stages of development. Both reproductive and embryological characteristics, such as presence of stomodeum in the larva, and ingestion of particles after hatching confirmed the planktotrophic veliger larvae of this species, consistent with the majority of sacoglossans from the Eastern and Northeast Pacific Oceans.**

KEY WORDS: *extra-zygotic yolk (EZY), spiral cleavage, reproductive mode, Sacoglossa*

Introduction

There are over 300 species of heterobranchs present in the Northeast Pacific Ocean, of which almost 70% have a known mode of development that can be either indirect, i.e. planktotrophic development, or direct, i.e. lecitotrophic and capsular metamorphic development (Goddard 2004; Goddard and Hermosillo 2008). Most species in this region develop from small eggs into planktotrophic larvae, only a few hatch as either lecithotrophic larvae or juveniles (Goddard and Hermosillo 2008). Worldwide, *Elysia* is the most diverse genus within the Sacoglossa, with 87 recorded species, including some cryptic species (Krug *et al.,* 2016). *Elysia* is distributed in both temperate and tropical regions, with two hotspots: the tropical Indo-Pacific and the Caribbean region (Jensen 1992, 2007). The Caribbean species have been well-studied in reproduc-

Abbreviations used in this paper: AV, animal-vegetal; BSA, bovine serum albumin; COI, cytochrome oxidase subunit I; DAPI, 4',6-diamidine-2-phenylindole; ECY, extra capsular yolk; ESS, effective sample size; EZY, extra-zygotic yolk; GTR, general time-reversible model; HKY, Hasegawa, Kishino, and Yano model; mtDNA, mitochondrial DNA; PBT, phosphate-buffered saline with Triton; PCR, polymerase chain reaction; SYM, symmetrical model.

***Address correspondence to:** Fernanda X. Oyarzún. Centro de Investigación en Biodiversidad y Ambientes Sustentables (CIBAS), Universidad Católica de la Santísima Concepción, Av. Alonso de Ribera 2850, Concepción, Región del Biobío, Chile. Tel: +56-9-7809-4542. E-mail: fernanda.oyarzun@ucsc.cl
#Note: These authors contributed equally to this work.

Supplementary Material (1 table + 2 movies) for this paper is available at: https://doi.org/10.1387/ijdb.200157fo

Submitted: 15 February, 2020; *Accepted:* 30 May, 2020 *Published online:* 26 August, 2020.

ISSN: Online 1696-3547, Print 0214-6282

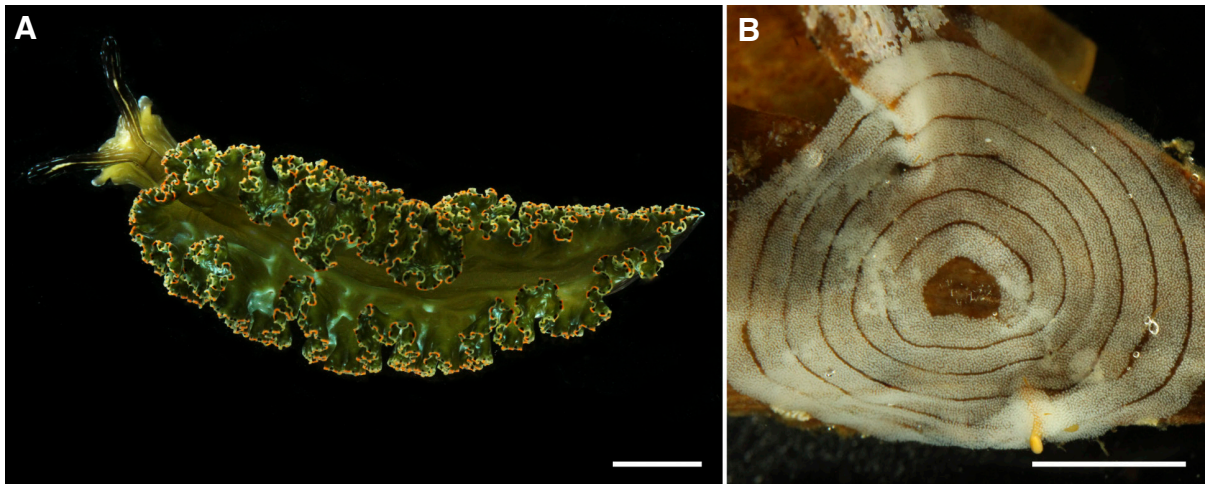

Fig. 1. *Elysia diomedea,* also known as the "Mexican Dancer". (A) *Ballenita's specimen of E. diomedea.* **(B)** *E. diomedea egg ribbon laid on the alga Padina sp. Scale bar, 1 cm.*

tive and phylogenetic aspects (Krug *et al*., 2016).

Elysia diomedea - earlier referred to as "dancers" by Berscht y Smith (1973) and as "Mexican dancer" in this paper because its coloration patterns that resemble traditional Mexican dance dresses - is a common sea slug species, originally described in 1894 by the malacologist Rudolph Berg (revised by Marcus, 1982). It is distributed along the east Pacific coast: from the Gulf of California (Bertsch and Smith, 1973) to the north of Peru (Uribe *et al*., 2013). *E. diomedea* is a common species in Ecuador, but besides two undergraduate theses focusing on food preferences and behavior (Chávez, 2012) and spatio-temporal abundance in the intertidal zone (Reyes, 2014), no other studies have been published on these Ecuadorian populations. We observed that populations of *E. diomedea* in Ecuador differed in color and adult size, raising questions about the delimitation of the species. Other sacoglossans have been shown to be cryptic species complexes (e.g., see Krug, 2007), and one *Alderia willowi* exhibited an interesting seasonal polyphenism in different populations, producing different proportions of planktotrophic and lecitotrophic larvae according to seasons and locality (e.g. planktotrophy was prevalent in winter and spring and lecitotrophy in summer and fall; Krug *et al*., 2012). Therefore, to delimit *E. diomedea*, we addressed whether the morphologically distinct populations share similar alleles using molecular data, and investigated the reproduction, embryology, and developmental mode of this species. *E. diomedea* represents an interesting species to study how different historical and ecological contexts can shape variation in reproduction and development of the species.

While some general aspects about *E. diomedea* reproduction have been studied, the patterns of spiral cleavage in early embryos of this species have remained understudied. Egg ribbons consisted of spiral-shaped gelatinous ribbons attached to rocks or macroalgae, and a 2 mm long segment of its ribbon contained 14 to 16 capsules, each of which contained 6 to 14 embryos (Bertsch and Smith, 1973). Goddard and Hermosillo (2008) reported that embryos hatched as planktotrophic larvae with no eyespots and an average shell length of 128±2.4 μm in a population in Bahía de Banderas, México.

Spiral cleavage is characterized by a diagonal cell division with a 45° shift respect to the animal-vegetal (AV) axis. This results in a spiral-like arrangement of the cells, that gives the name 'Spiralia' to a clade of eight major animal groups, including Annelida, Nemertea, Plathyelminthes and Mollusca (Chávez *et al.,* 2017; Martin-Duran and Marletaz, 2020). Despite being a synapomorphy of around 10% of the known animal species, spiral cleavage remains one of the most under-investigated cleavage patterns (Martin-Duran and Marletaz, 2020). Within Spiralia, the clade Heterobranchia represents an interesting group to study the plasticity in early embryonic cell divisions because deviations from the typical formation of the four equal quadrants have been documented to occur in many orders, including Pteropoda, Umbraculida and Anaspidea, where an unequal cleavage takes place (Chávez *et al.,* 2017; Van den Biggelaar and Haszprunar 1996). In addition, and interesting but rare case of reproductive plasticity —interspecific variation in the type of larvae produced (e.g. poecilogony)— has been well-documented in nine species of sacoglossan gastropods: *Alderia willowi, Costasiella ocellifera, Calyptraea lichen, Elysia pusilla, E. zuleica, E. chlorotica, E. velutinus, E. subornata and E. papillosa* (reviewed in Vendetti *et al.,* 2012; McDonald *et al.,* 2014; De Jesus 2018)

Here we confirm the presence of *E. diomedea* (Fig. 1A) in Ecuador using two mitochondrial fragments while testing for possible cryptic species based on the reported variation in morphology among three localities. Then we document in detail the early development for the species which has not been previously examined. We discuss the cleavage patterns of *E. diomedea* in relation to what has been reported for other heterobranchs and caenogastropods. Finally, we confirm the reported planktotrophy of the *E. diomedea* larva. We hope our study will help generate additional comparative studies in the genus *Elysia* in the future, increasing our understanding of geographic and reproductive variation in the life history traits of heterobranchs.

Results

Collection of specimens

The mean length of individuals collected in Mompiche was 3.2 ~ 0.53 cm (n = 14), 4.6 ~ 0.6 cm (n = 7) in Cabuya, and 8.4 ~ 0.82 cm (n = 10) in Ballenita. The rocky substrate differed at each site: in Mompiche the platform was made up of black rock, in Cabuya

by a dark sandstone and in Ballenita for a brighter sandstone; highest abundance was found in the Mompiche.

Molecular confirmation of Elysia diomedea

The specimens analyzed here were successfully identified as *Elysia diomedea* and were grouped in a clade with high support (posterior probability > 0.98; bootstrap > 75) as seen in Fig. 2. The tree topologies for Bayesian and maximum likelihood phylogenies were similar, except for nodes with weak support (posterior probability and bootstrap <50) (Fig. S1). The molecular fragments analyzed in this study showed no population structure among individuals that could explain the morphological differences described above.

Reproduction and development of E. diomedea in laboratory conditions

In the laboratory only individuals collected from Ballenita reproduced, from which five egg ribbons were obtained over a period of three months (Fig. 1). After twelve weeks in captivity, adult individuals began to shrink until they disappeared.

Before copulation, individuals showed an increase in activity. They moved around the aquarium and sought out partners by making multiple contacts with each other. Spawning time varied between three and five hours, depending on the length of the ribbon.

TABLE 1

EMBRYONIC DEVELOPMENTAL STAGES OF *ELYSIA DIOMEDEA* WITHIN THE EGG CAPSULE AT AMBIENT TEMPERATURE (18-20ºC)

Stage of development	Time
Oviposition	0h
1st cleavage (2 cells)	40 min
2nd cleavage (3 cells)	1 h
3rd cleavage (4 cells)	1,5 - 2 h
4th cleavage (8 cells)	4 - 5 h
5th cleavage (12 cells)	6 - 7 h
6th cleavage (16 cells)	8 - 9 h
Stereoblastula	24 h
Gastrula	4 days
Trochophore	6 days
Hatching	10 - 11 days

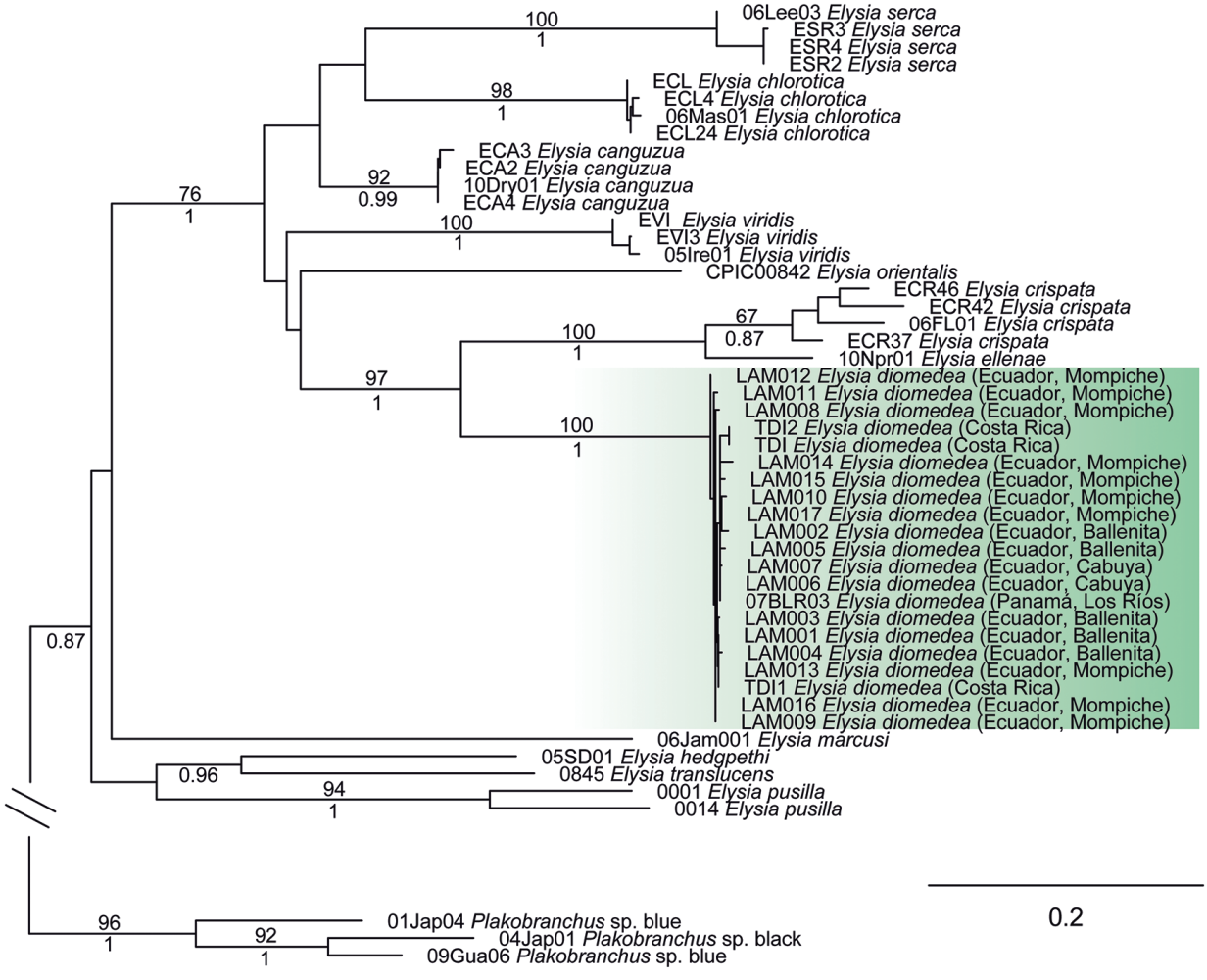

Fig. 2. Maximum Likelihood phylogenetic tree of *Elysia* genus that includes the three sampled populations of Ecuador. *Tree topology corresponds to the maximum likelihood analysis of concatenated DNA sequences (COI, 16S; 1116 pb). Bootstrap supports (>50) and posterior probabilities (>0.5) are above and below each branch respectively. GenBank accession numbers are shown in Appendix 1 (see Supplementary Material).*

The egg ribbons consisted of a white anti-clockwise spiral ribbon formed by a gelatinous matrix in which the capsules were embedded. They were placed attached to the walls of the aquariums and algae *Padina* sp. and *Codium* sp. (Fig. 2B). The diameter of the egg ribbon was 3.0 ± 0.6 cm (N = 5), the width of the ribbon being 1 mm and the length of 35.2 ± 10.5 cm (N = 5). The number of capsules per mm^2 of egg ribbon was 98 ± 9.21 (N = 5), with only one embryo per capsule. Zygotes measured 62.06 ± 6.93 μm (N = 20). Intracapsular development of embryos throughout the egg ribbon was generally synchronous; however, <25% of embryos were observed to develop in asynchrony.

Embryos cultured at room temperature (21.4 ± 2.35°C; n=5) developed to the trochophore stage 6 days after oviposition, and planktotrophic larvae hatched from their capsules after 11 days (Table 1). Immediately after oviposition, polar bodies were observed attached to the 1- and 2-cell embryos (Fig. 3A). Polar bodies remained near the animal pole of embryos (Fig. 3A-B). An unequal cleavage occurred in the early embryo 40 minutes after oviposition (Table 1), which resulted in two blastomeres of different sizes: AB being slightly smaller than CD. During this stage, the polar bodies localized at the animal pole along the cleavage furrow of both cells (Fig. 3B; Fig. 4A). The next embryonic cleavages occurred in blastomeres AB and CD around 1h-2h after oviposition. We observed a brief 3-cell stage in a few embryos, in which cells were not completely round, but oval (Fig. 3C). Cleavage of blastomere CD was observed between 1.5 to 2 hours after oviposition (Table 1), resulting in an embryo with four blastomeres (Fig. 3D and 4B). Two of them (A and B) were smaller, and the other two (C and D) were larger. The 8-cell stage occurred within 4-5 hours (Table 1), and the macromeres (1A, 1B, 1C and 1D) and micromeres (1a, 1b, 1c and 1d) could be clearly identified (Fig. 3E). The 12-cell stage occurred between 6-7 hours after oviposition (Table 1). At this stage a second quartet of large micromeres was formed (2a, 2b, 2c and 2d) (Fig. 3F), whereas the first quartet of micromeres (1a, 1b, 1c and 1d) remained uncleaved. The 16 cells embryo occurred between 8 and 9 hours after oviposition (Table 1). This stage was reached after the first quartet of micromeres cleaved resulting in the formation of a new quartet of micromeres (1a^2, 1b^2, 1c^2 and 1d$^{2)}$ near the animal pole, whereas the the second quartet (2a, 2b, 2c and 2d) and macromeres (2A, 2B, 2C and 2D)

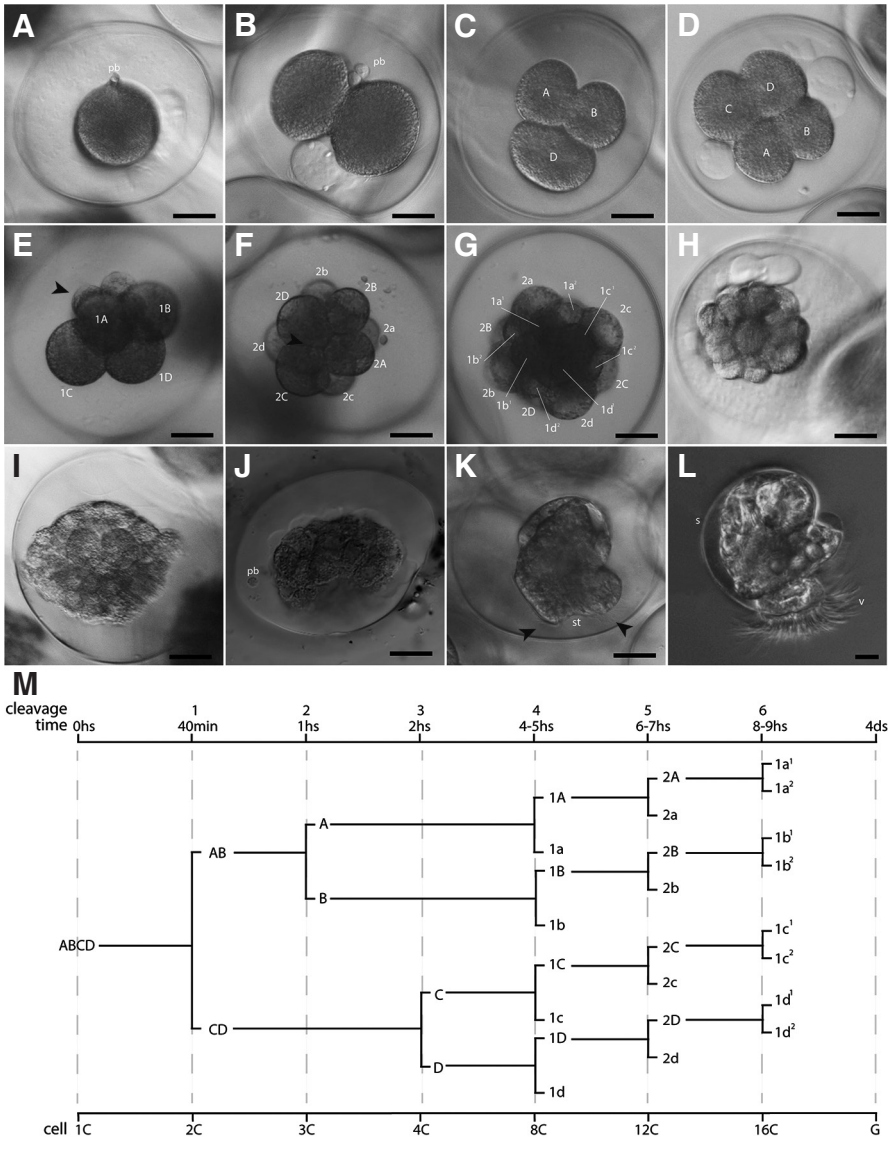

Fig. 3. Early development of *Elysia diomedea.* (A) *Zygote stage or 1-cell embryo, a polar body is visible (pb).* **(B)** *First cleavage, the polar bodies are located on the cleavage furrow of the cell.* **(C)** *Transient stage of three cells was observed in some embryos; the blastomeres A and B have completely cleaved and the CD blastomere remains uncleaved.* **(D)** *Embryo at the 4-cell stage, blastomeres C and D are larger, and blastomeres A and B are smaller.* **(E)** *Embryo at the 8-cell stage viewed from the vegetal pole, micromeres (arrowhead) are behind the macromeres 1A, 1B, 1C, and 1D* **(F)** *Embryo at the 12-cell stage viewed from the vegetal pole, the second quartet of large micromeres (2a, 2b, 2c, 2d) are shown behind the four macromeres (2A, 2B, 2C, 2D), whereas the first quartet of micromeres remain uncleaved at the animal pole and are not visible* **(G)** *Embryo at 16-cell stage viewed from the animal pole, the first quartet of micromeres has cleaved to form micromeres 1a1, 1b1, 1c1, 1d1, 1a2, 1b2, 1c2, 1d2, whereas the large micromeres (2a, 2b, 2c, 2d) and macromeres (2A, 2B, 2C, 2D) remain uncleaved.* **(H,I)** *Stereoblastula stages (≥20 cells), movements of epiboly have started.* **(J)** *Embryo at the gastrula stage, blastopore is shown below and a polar body (pb) has detached from the embryo.* **(K)** *Trochophore larvae, the intestine has formed completely, and the embryo presents cilia (arrowheads) that allow it to move inside the capsule.* **(L)** *Newly hatched larva, left lateral view.* **(M)** *Cell lineage of early cleavage stages in E. diomedea embryos, note the asynchronies during early development. Extra zygotic yolk (EZY) bodies are shown in (A-B), (D) and (H). Scale bar, 25 μm.*

remained uncleaved (Fig. 3G). Stereoblastula stages (≥20 cells) were reached after 24 hours (Fig. 3H; Table 1). Epiboly caused the micromeres to multiply and surround the vegetal macromeres (Fig. 3I-J). Gastrulation occurred four days after oviposition (Table 1). During this stage, the embryo presented a characteristic gastrula shape with a small cleft at the vegetal pole, the blastopore (Figs. 3J,4E), which later formed the mouth (Fig. 3K and 4E-G). In early embryos upto the gastrula stage of two egg ribbons (of 5 analyzed in total) we observed the occurrence of one or more extra-embryonic globular structures of approximately 25 μm in diameter that came into contact with blastomeres of the embryo. These structures often contained inner spheroid vesicles (<4 μm in diameter) similar to lipid droplets (Fig. 3 B-D, H). After fixation these structures disappeared, and we could not follow the fate of these structures in later stages of development.

The cilia became visible when the embryo reached the trochophore stage about six days after oviposition (Fig 3K, black arrows). At this stage, the gut tube was completely formed and opened outward, giving rise to the stomodeum, or mouth, and the initial shell was possible to distinguish (Fig. 3K: Fig. 4G). At the trochophore stage, the ciliary bands of the embryo facilitated its movement inside the capsule. Nine days after oviposition, a spiral shell with ¾ a twist (a Type 1 shell of Thompson, 1960), can be observed (Fig. 3L). Thereafter, the statocysts and the visceral mass became visible. By day ten, a fully functional retractor muscle enabled the trochophore to retract into its shell and an increased movement was observed within the capsule. Active rapid and rotational movements of the trochophore facilitated the capsule to deteriorate and break (Supplementary video 1). Veliger larvae hatched from the egg ribbon within the following 20 to 30 hours. Veligers had a characteristic Type 1 shell of 151.02 μm diameter, with no visible eye-spot, with active cilia and a fully developed and functional buccal apparatus and gut. During our observations of the newly hatched veligers, we documented a larva capturing and ingesting a particle that was in the media (Supplementary video 2).

Discussion

Our phylogenetic analysis (Fig. 2) was consistent with that reported by Krug *et al.,* (2016) for the genus *Elysia* and confirmed the presence of *E. diomedea* in Ecuador. In addition we did not find genetic structure that might explain the morphological differences found among the individuals of the three sites and examine this morphological variation in greater detail elsewhere (Moreano-Arrobo *et al*., in preparation).

In our study, the spawning time of *E. diomedea* depended on the length of the ribbon, which is consistent with species such as *Stylocheilus striatus* and *Aplysia californica* (Horwitz *et al.,* 2017; Ferguson *et al.,* 1989). The duration of oviposition varies within the heterobranchs and can take between one to ten hours (Hadfield and Switzer-Dunlap, 1984). Oviposition by *E. diomedea* occurred on the walls of the aquariums and on the alga *Padina* sp., consistent with observations of other species of *Elysia* (Chávez 2012; Reid 1964; Greene 1968; Hamatani 1960; Hagerman 1970; Rahat

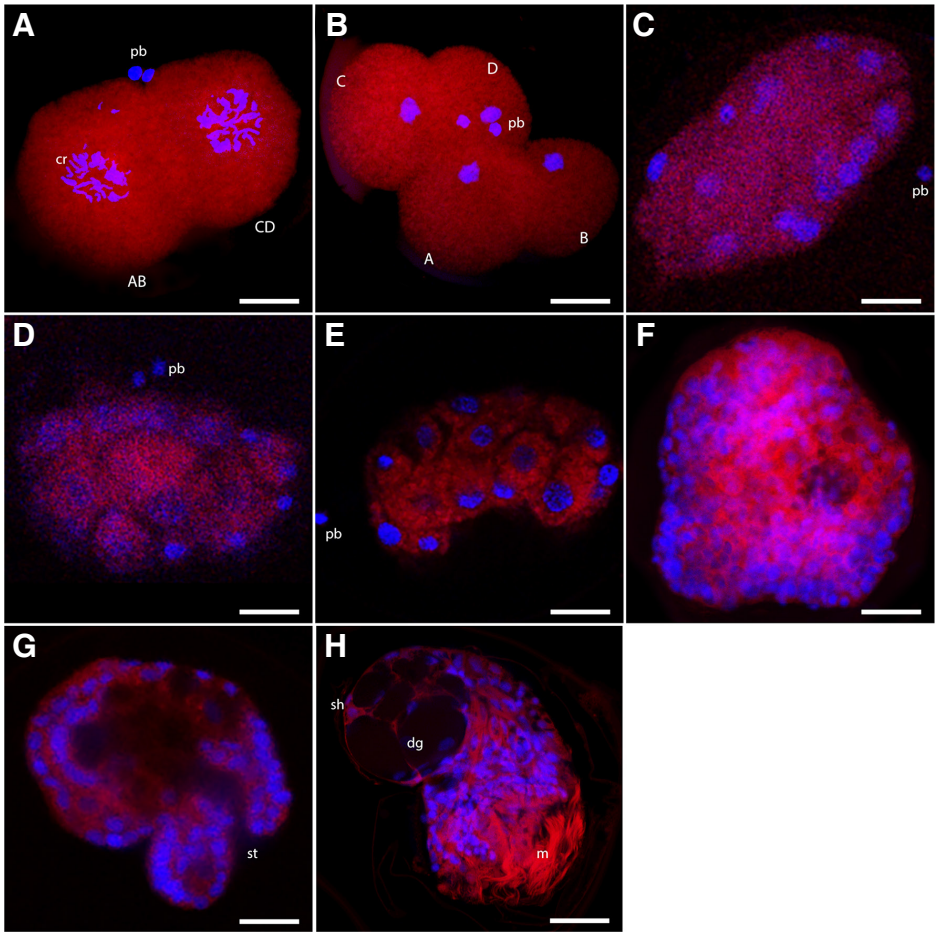

Fig. 4. Confocal microscopy in *Elysia diomedea* reveals anucleated extra-embryonic bodies to confirm the occurrence of extra zygotic yolk (EZY) embryos, as well as the presence of the intestine in the trochophore confirming its planktotrophic mode of development. (A) *First cleavage, the polar bodies (pb) remain close to the cleavage furrow., and the chromosomes of the blastomeres begin to decondense.* **(B)** *Embryo at 4-cell stage, the polar bodies (pb) are visible* **(C,D)** *Stereoblastula stages (>20 cells) undergoing epiboly, larger cells (macromers) and smaller ones (micromeres) are visible, and the polar bodies (pb) remain closely associated.* **(E)** *Gastrula stage, a typical gastrula stage is observed in which the epibolic movements have occurred and the blastopore has been formed (bp, arrowhead); polar bodies (pb) have detached from the embryo.* **(F)** *Gastrula, external view; the surface of the gastrula is highly nucleated, and the internal cavities can be observed.* **(G)** *Trochophore larva, internal view; the stomodeum (st) is observed at the opening of the intestine* **(H)** *Hatched veliger, the shell (sh), the digestive gland (dg), and musculature (m) of the foot, mantle and velum are identified. Actin filaments are shown in red (Phalloidin) and the nuclei and polar bodies in blue (DAPI). Two photon confocal microscopy was used in (A,B,F,H), whereas a normal confocal microscopy was used to generate (C-E,G). Scale bar, 25 μm.*

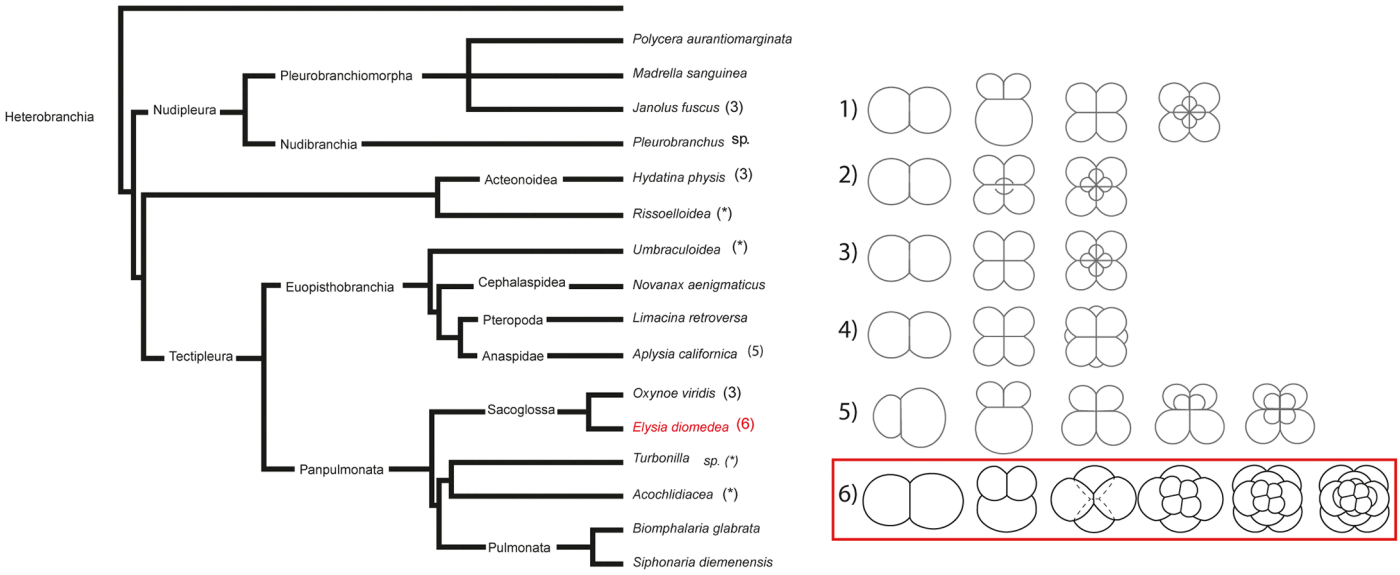

Fig. 5. Comparison of cleavage patterns observed in *Heterobranchia*. *Phylogeny based on molecular analyses of the group Heterobranchia (Zapata et al., 2014, 2015). Previously described cleavage patterns of model species are used to exemplify the high variation found within the group (Chavez, et al., 2017). The newly described cleavage pattern observed in E. diomedea (6) noticeably differs from the rest of the group by exhibiting slightly unequal and asynchronous early cell divisions. The 8-cell embryos presented a typical formation of the first quartet of micromeres (1a, 1b, 1c, and 1d) that were smaller in cell size than the macromeres, whereas the 12-cell embryo showed the formation of the second quartet of micromeres (2a, 2b, 2c, and 2d) with unusually large cell size, i.e. as large as the macromeres (2A, 2B, 2C, and 2D).*

1976; Ellingson and Krug 2006; Krug *et al.,* 2016) and with the description of Bertsch and Smith (1973) for *E. diomedea*.

We found that *E. diomedea* deposited one egg per capsule. This contrasts with the 6-14 embryos per capsule reported by Bertsch and Smith (1973) for this species from the Gulf of California, which could represent a misidentification of the species. However, more interestingly, this difference raises questions worth pursuing about potential intra-specific variation in this trait and possible genetic variation in *E. diomedea* across its range.

The patterns of blastomere formation during early development in *Elysia diomedea* uncovered a variation in size and timing of cleavages compared to other heterobranch species. An unequal first cleavage is a condition which deviates from the stereotypical equal cleavage of most heterobranchs and other molluscs (Chavez *et al*., 2017). However, in the Heterobranchia, a very pronounced and characteristic unequal first cleavage has been documented in Anaspidea (Saunders and Poole 1910; Heyland *et al.,* 2011; Lee *et al.,* 2014; Chavez *et al.,* 2017), and less pronounced unequal first cleavages have been observed in some species of Sacoglossa, including two species of *Limapontia* (Chia, 1971) and *E. diomedea* (this work). A three-cell stage embryo (Fig. 3C) reported here for the sacoglossan *E. diomedea,* presumably generated by a brief delay in the cleavage of blastomere CD over blastomere AB, was carefully examined previously by one of our labs in the anaspid *Aplysia* cf. *californica* (Chávez *et al*., 2017). Whether this stage actually occurs in only a few embryos, or whether all embryos pass through this stage very rapidly remains to be determined. At the 3-cell stage, Besides these two species that belong to Tectipleura, another 3-cell stage embryo has been previously reported in the Nudipleura, specifically in the nudibranch *Polycera aurantiomarginata* (Martínez-Pita *et al*., 2006). Because we observed that the 3-cell stage embryos were preceded by a first unequal cleavage in both species we examined (to a larger degree in *A.* cf. *californica* and to a lesser degree in *E. diomedea*), we returned our attention to the micrographs taken by I. Martínez-Pita and collaborators (2006) of the 2-cell stage embryos in *P. aurantiomarginata* that were described by them to present an equal and holoblastic cleavage. Here we noticed that one of the 2-cell blastomeres was indeed slightly smaller (presumably AB) than the other blastomere (presumably CD) (see Fig. 2C in Martínez-Pita *et al*., 2006). Thus, we suspect that the 3-cell stage embryo observed across distantly related heterobranchs may have convergently evolved as a consequence of an uneven deposition of cytoplasmic contents into blastomere AB and blastomere CD, resulting from an unequal first cleavage. It is important to search for unequal first cleavers and 3-cell stage embryos in other species of heterobranchs. In general, variability of cleavage patterns in many heterobranchs remains poorly documented and it is important to describe all the variability in the group to begin to understand the consequences of early developmental patterns in their evolution.

The formation of a large second quartet of micromeres in early 12-cell embryos of Sacoglossa is unusual compared to other spiralians. While the *E. diomedea* 8-cell embryos presented a typical formation of the first quartet of micromeres (1a, 1b, 1c, and 1d) that were smaller in cell size than the macromeres, the 12-cell embryo showed the formation of the second quartet of micromeres (2a, 2b, 2c, and 2d) with unusually large cell size, i.e. as large as the macromeres (2A, 2B, 2C, and 2D). Similarly large cells in the second micromere quartet were also shown to occur in other species of Sacoglossa, including two species of *Limapontia* (Chia, 1971). Typically, second quartet micromeres of spiralian embryos show nervous system fates, as well as ectodermal fates in the anterior trunk region of the prototroch or at the prototroch of the larva (Rabinowitz and Lambert 2010; Lyons and Henry

2014). Because micromere quartets show conserved ectodermal and ectomesodermal fates in larval domains across spiralians, it is interesting to speculate how the enlarged cells in the second quartet of the 12-cell embryo of sacoglossans may have contributed to particular expansions of larval domains. Other spiralians, including several species of nemerteans and sipunculids, have been reported to display enlarged cells in the animal micromere quartets (Hoffman 1877; Wilson 1900; Henry and Martindale 1997; Maslakova *et al.,* 2004; von Döhren 2015). Many open questions remain about the extent that changes in blastomere size can affect adult and larval phenotypes.

Although most opisthobranchs develop using their own zygotic nutrient reserves stored in the cytoplasm (Clark and Jensen 1981; Hadfield 1985), extra zygotic yolk (EZY) of several species of opisthobranchs with distinct developmental modes, including indirect developers (i.e. planktotrophic development) and direct developers (i.e. lecitotrophic and capsular metamorphic development), can serve as extra-embryonic food reserve (Chia 1971; Bridges 1975; Clark and Goetzfried 1978). EZY can come in different forms in the Heterobranchia, for example: (i) variable ribbons of extra capsular yolk (ECY) that are in direct contact with egg capsules and zigzag through the egg ribbons of the *m*any species of *Elysia*, regardless their developmental modes (Krug *et al.,* 2016; Thompson and Salghetti-Drioli 1984; Vendetti *et al.,* 2012); (ii) unusually large polar bodies (18 μm in diameter, in comparison to ova of 100 μm) packed with yolk droplets inside the egg capsule of the planktotrophic aeolid nudibranch *Cuthona lagunae* (Goddard, 1991); or (iii) refractyle and granular intracapsular bodies of different size, including of small vesicles lining the inner wall of the egg capsule in the direct-developing sacoglossan sea slug *Costasiella lilinae* (Clark and Goetzfried 1978; Goddard 1984), others of a third the size of the ova (49 μm in diameter, in comparison to ova of 144-157 μm) in the direct-developing (i.e. capsular metamorphic) aplysiid seahare *Phyllaplysia taylori* (Macginitie 1935; Bridges 1975). It has been documented that EZY gradually becomes fragmented into smaller particles during embryonic intracapsular development and often disappear before larvae hatch, presumably due to its ingestion by the developing veliger in *Cuthona lagunae* (Goddard, 1991), or by storage of these particles in the veliconch in *Phyllaplysia taylori* (Bridges, 1975). *S*ome controversy remains whether embryos are capable of ingesting EZY, as suggested for several sacoglossan species (Clark and Goetzfried 1978; Clark and Jensen 1981). Both the absence of nuclei (Fig. 4) and relatively large size of the extraembryonic globular structures described here in *E. diomedea* (Fig. 3, Fig. 4) show that these structures correspond to refractile intracapsular bodies. We could clearly differentiate these structures from the polar bodies, which clearly showed the presence of nuclei (Fig. 4). The intracapsular bodies measured nearly half the size of the ova (approximately 25 μm in diameter, in comparison to ova of 62 μm), and we did not observe a change of size during the early cleavage stages of development, suggesting embryonic ingestion was absent at least in these very early stages of development. Unfortunately, we could not follow the fate of these structures any further and they disappeared after fixation preventing any further analyses. As has previously been observed in other opisthobranchs, ECY bodies are dispensable and vary between and within capsules (Chia 1971; Hadfield 1984). Intracapsular bodies in *E. diomedea* also showed intercapsular variation, and at first glance all five *E. diomedea* egg ribbons developed invariably, regardless of the presence of refractyle intracapsular bodies. However, intercapsular variation in *E. diomedea* opens the possibility that varying larval food supplies can be adjusted maternally in response to environmental variation, food shortage or other stressors such as pollution (Ellingson and Krug, 2006). Furthermore, we could speculate that EZY provisioning mechanisms may have large effects on the embryonic and larval developmental modes of opisthobranchs. EZY can be readily incorporated into the zygote or embryo of a planktotrophic opisthobranch ancestor, evolving larger size, which could eventually transition into lecithotrophy. Alternatively, EZY in capsules with multiple eggs can produce internal variation of embryonic and larval size during development in the intracapsular environment serving as pre-adaptations to poecilogony. It is essential that future studies explore the origin and fates of EZY, its chemical composition, and the extent that EZY provisioning is affected by different environmental conditions or nutritional levels of the parent.

E. diomedea larval development, size, and behaviors displayed features of planktotrophy. Thompson (1967) suggested that the size of the uncleaved embryos, the number of embryos per oviposition, the diameter of the larval shell, and the relatively short embryonic period were characteristic of planktotrophic larvae in heterobranchs. *E. diomedea* showed some of the largest egg ribbons registered within the *Elysia* genus with probably some of the highest numbers of reported eggs per oviposition (~39,000 eggs). Small and numerous eggs are generally associated with planktonic organisms (Allen and Pernet, 2007). The size of the shell at hatching (151.02 μm) was also consistent with planktotrophic larval shell (100-160 μm) (Vendetti *et al*., 2012). Most importantly and as ultimate proof for a feeding or a planktotrophic larva, we show here that the larvae of *E. diomedea* hatched with a fully developed buccal apparatus and gut, and that they can feed on particles immediately after hatching using their active cilia (Supplementary video 2).

Conclusions

1) First molecular identification of *Elysia diomedea* in Ecuador. Three populations that present different morphological characteristics belong to the same species and have no genetic structure.

2) Analyses of *E. diomedea* early spiral development and its comparison to other heterobranchs reveals variations in blastomere size, timing of cleavages, and presence of extra zygotic yolk (EZY) bodies that could have interesting implications for the evolution of larval and adult body types, as well as developmental modes.

Materials and Methods

Collection of specimens

A total of 46 specimens were collected manually from intertidal rocky shores in low tide following the Camacho-García *et al.,*2005) field guide. Specimens were collected in three localities on the Ecuadorian coast: 1) Ballenita, Santa Elena Province (2° 12' 21.4" S, 80° 52' 50.98" W), 2) la Cabuya, Manabí Province (0° 02' 58.83" S, 80° 08' 35.59" W), and 3) Mompiche, Esmeraldas Province (0° 30' 25.63" N, 80° 01' 47.66" W). Host alga *Codium* sp. and *Padina* sp. were taken to feed adults for posterior culture procedures. Seventeen individuals were stored in alcohol in the field for subsequent molecular confirmation of *Elysia diomedea* species.

Molecular confirmation of Elysia diomedea

Total genomic DNA was isolated from muscle tissue samples using

the DNeasy® Blood and Tissue Kit (Qiagen, Inc., Valencia, CA). Two mitochondrial (i.e. the large ribosomal subunit rRNA, 16S and Cytochrome Oxidase subunit I, COI) gene fragments were amplified. The 16S (420 bp) was amplified using 16Ssar 5'-CGC CTG TTT ATC AAA AAC AT-3' and 16sa 5'-CTC CGG TTT GAA CTC AGA TC-3' (Palumbi, 1996); the COI (658 bp) using LCO1490 5'-GGT CAA ATC ATA AAG ATA TTG G-3' and HCO2198 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' (Folmer *et al.*, 1994); For 16S fragment, the PCRs were conducted in 25 μL volumes containing 67 mM Tris-HCl pH 8.8, 16 mM $(NH_4)2SO_4$, 3 mM $MgCl_2$, 0.2 mM of each dNTP's, 0.6 μM of each primer, 0.4 mg/mL of Bovine Serum Albumin (BSA), 1.5 U/μl Taq DNA polymerase (Fisher®), and 20 ng/ μL of genomic DNA. Thermal cycling was performed in an Eppendorf Mastercycler EP gradient S thermocycler with the following parameters: 95° C for 300 s, followed by 35 cycles for 16S. PCR product were visualized using ethidium bromide stain, then forward and reverse strands were sequenced, using an ABI 3730xl BigDye Terminator Cycle Sequencing 3.1 (Applied Biosystems) standard protocol (Macrogen Inc., Seoul, Korea). Sequences were deposited in the GenBank database under the following accession numbers (COI: MT511640-MT511655; 16S: MT509960-MT509976).

For the molecular confirmation, we obtained sequences from GenBank of 73 different species in the genus *Elysia*, which included three other *E. diomedea* specimens from Costa Rica and one from Panamá (Table S1). These sequences were aligned with *E. diomedea* DNA sequences generated in this study. For the outgroup, we added three species of *Plakobranchus* genus (Krug *et al.*, 2016). Sequence alignment was conducted in Geneious v11.1.5 (GeneMatters Corp) using the ClustalW alignment algorithm. Finally, we used the Mesquite v3.51 software (Maddison and Maddison, 2015) for handling sequences. Final alignment was adjusted by eye.

Because it is likely that variation in each of our sampled genes (or codon positions within protein coding genes) were shaped by different evolutionary processes, we partitioned the data according to gene and codon position to analyze each partition under separate models of evolution. We used software PartitionFinder v.1.1.1 (Lanfear *et al.*, 2012) to simultaneously estimate both the best-fit model for each partition and the best partition strategy for our data (i.e. 16S and 1st, 2nd, 3rd position for COI codons). No partitions were made in 16S because this is not a coding gene. The best model for our four partitions were: 16S gene (GTR + I + G), 1st position for COI (HKY + G), 2nd position COI (SYM + G), and 3rd position for COI (GTR + I + G).

Phylogenetic analyses were inferred using Bayesian inference and Maximum-Likelihood. Bayesian analyses were conducted on MrBayes 3.2 software (Ronquist *et al.,* 2012) running four parallel runs of the Metropolis coupled Monte Carlo Markov chain for 30×10^6 generations. Each run had four chains with a temperature of 0.1. For each analysis, the chain was sampled every 1,000 generations. The first 10% of sampled trees were discarded as burn-in and the remaining trees were used to estimate the Bayesian tree, posterior probabilities, and other model parameters. Tracer software version 1.7 (Rambaut *et al.,* 2018) was used to confirm convergence and stationarity of the parameter estimates using an ESS threshold of 200. Maximum likelihood analyses were conducted using GARLI 2.0 (Zwickl, 2006) with default settings. Support was evaluated using 100 bootstrap replicates with each replicate terminated after 5,000 replications without improvement in topology. The consensus tree was obtained with the software Mesquite using the majority rule of 50%.

Reproduction and development of E. diomedea in laboratory conditions

Live slugs and algae were transported and maintained under controlled conditions (salinity of 30 ± 1 ppt; 18-30°C; 12:12 h light/dark cycles) in aquaria in the Laboratory of Developmental Biology 113 at the Pontificia Universidad Católica del Ecuador - PUCE. No special treatment was done for individuals to reproduce, and oviposition occurred in the laboratory over three months. As soon as oviposition had occurred, egg ribbons were taken, measured and described using a light SZX2-ILLT stereomicroscope. Egg ribbons were retrieved and kept in 20 ml petri dishes with sterile seawater with salinity of 30 ± 1 ppt; 12:12 h light/dark cycles, and temperature range of 18-20°C. Embryos were fixed in 4% paraformaldehyde for 12 h at 4°C. Then, they were rinsed three times every 10 minutes with Phosphate-buffered Saline (PBS) with Triton or PBT (0.1% Triton-X in PBS) and stored at 4°C in glass vials with PBS 1X. The diameter of uncleaved eggs (zygotes) was measured from random samples (n=30) using SZX2-ILLT stereomicroscope Lumera software. In five egg ribbons, hatching of the larvae occurred naturally. From these hatching events, larvae were collected and their shell length was measured from the forward edge of the aperture to the rear of the shell, general characteristics observed, photographed and recorded.

Live embryos and larvae were observed, recorded and photographed with the inverted ZEISS TELAVAL 31 microscope, Infinity 1 camera and the Lumenera's Infinity Capture software®. For fluorescent microscopy, the embryos were fixed and labeled with 4',6-diamidine-2-phenylindole (DAPI) and the non-immunological probe Phalloidin (Adams y Pringle, 1991). The embryos were placed in a glass slide with a drop of autoclaved glycerol (70%), and then we put a coverslip with plasticine between the slip and the slide as spacers. Next, the coverslip was sealed, and the slides were placed in dark containers. The confocal microscopy photographs were taken using a Leica DM6 system in the Brown Lab at the University of Sao Paulo USP, Brasil and in the Advanced Microscopy Center (CMA BIO-BIO) of the University of Concepción, Chile, with a two-photon confocal spectral microscope, LSM780 NLO Zeiss, and edited in the ImageJ® and Adobe Photoshop CS6 software.

Acknowledgments

This manuscript was completed after the first co-author Oscar Pérez passed away. The rest of the authors would like to express their gratitude for his inexhaustible curiosity and love for science, for being a great mentor and colleague and for the essential role he played to make this article possible. We are indebted to the Universidad de Los Lagos and the Centro i~mar for their hospitality and help with equipment and foundation for the molecular analyses. We thank the Centro de Microscopía Avanzada, CMA Bío-Bío, as well as Mark Martindale from the Whitney Laboratory for Marine Bioscience, University of Florida for giving his opinion about the embryonic structures that provided us with the initial hint that led us to identify the EZY bodies in the early embryos. We also thank Marcel Caminer for his help with phylogenetic analyses. We thank Jeffrey Goddard and two anonymous reviewers who helped to greatly improve the manuscript. This work was funded and supported logistically by the Pontificia Universidad Católica del Ecuador. FDB was supported by FAPESP grants 2019/06927-5, 2015/50164-5, and 2018/50017-0. CBC-A was supported by Nucleo Milenio INVASAL funded by Chile's government program, Iniciativa Científica Milenio from Ministerio de Economía, Fomento y Turismo and Fondecyt 11180897.

References

ADAMS A E M and PRINGLE J R (1991). Staining of actin with fluorochrome-conjugated phalloidin. *Methods Enzymol* 194: 729-731.

ALLEN J D and PERNET B (2007). Intermediate modes of larval development: Bridging the gap between planktotrophy and lecithotrophy. *Evol Dev* 9: 643–653.

BASS A L (2006). Evolutionary genetics of the family Placobranchidae (Mollusca: Gastropoda: Opisthobranchia: Sacoglossa), PhD Thesis, University of South Florida, USA, 144p.

BASS A L and KARL S A (2006) Molecular phylogenetic analysis of genera in the family Plakobranchidae (Mollusca: Opisthobranchia: Sacoglossa). *Rec West Aust Mus* 69: 61–68.

BERTSCH H and SMITH A A (1973). Southwestern Association of Naturalists Observations on three Opisthobranchs (Mollusca: Gastropoda) of the La Paz Area, Baja. *Southwest Nat* 18: 165–176.

BRIDGES C (1975). Larval development of *Phyllaplysia taylori* Dall, with a discussion of development in the Anaspidea (Opisthobranchiata: Anaspidea). *Ophelia* 14: 161–184.

CAMACHO-GARCÍA Y E, GOSLINER T M and VALDÉS Á (2005). *Field Guide to the Sea Slugs of the Tropical Eastern Pacific.* California Academy of Sciences, San Francisco, CA, USA. 129p.

CHÁVEZ Y (2012). Estudios experimentales de comportamiento en la babosa marina fotosintética *Elysia diomedea* (Opisthobranchia: Sacoglossa) ante distintas condiciones de luz y variedad de dieta (undergraduate thesis). Pontificia Universidad Católica Del Ecuador. Quito, Ecuador.

CHÁVEZ-VITERI Y E, BROWN F D and PÉREZ O D (2017). Deviating from the Norm: Peculiarities of *Aplysia* cf. *californica* early cleavage compared to traditional Spiralian models. *J Exp Zool B Mol Dev Evol* 328: 72–87.

CHIA F (1971). Oviposition, fecundity, and larval development of three sacoglossan opisthobranchs for the Northumberland Coast, England. *Veliger* 13: 319-325.

CHIA F, GIBSON G and QIAN P (1996). Poecilogony as a reproductive strategy of marine invertebrates. *Oceanol Acta* 19: 203-208.

CHRISTA G, DE VRIES J, JAHNS P and GOULD S B (2014). Switching off photosynthesis: The dark side of sacoglossan slugs. *Integr Comp Biol* 7: 3–6.

CLARK K and GOETZFRIED A (1978). Zoogeographic influences on development patterns of North Atlantic Ascoglossa and Nudibranchiata with a discussion of factors affecting egg size and number. *J Molluscan Stud* 44: 283–294.

CLARK K and JENSEN K (1981) A comparison of egg size, capsule size, and development patterns in the order Ascoglossa (Sacoglossa) (Mollusca: Opisthobranchia). *Int J Invertebr Repr Dev* 3: 57-64.

CONTRERAS R and PÉREZ M (2010). Desarrollo embrionario y larval temprano en *Diaulula punctuolata* (d'Orbigny, 1837) (Nudibranchia: Doridacea) en condiciones de laboratorio. *Amici Molluscarum* 18: 13-20

DE JESÚS J (2018). *Unusual patterns of population genetic structure explained by three new cases of poecilogony and atypical larval behavior in tropical sea slugs* (Graduate thesis). California State University. Los Angeles, USA.

EGÜEZ A, GAONA M and ALBÁN A. (2017). Mapa Geológico de la República del Ecuador. Ministerio de Minería e Instituto Nacional de Investigación Geológico Minero metalúrgico. Available at: https://www.geoinvestigacion.gob.ec/mapas/nuevo%201M/GEOLOGICO_2017_1M.pdf

ELLINGSON R and KRUG P (2006). Evolution of poecilogony from planktotrophy: cryptic speciation, phylogeography, and larval development in the gastropod genus *Alderia*. *Evolution* 60: 2293-2310.

FERGUSON G P, TER MAAT A, PARSONS D W, PINSKER H M (1989). Egg laying in *Aplysia*: I. Behavioral patterns and muscle activity of freely behaving animals after selectively elicited bag cell discharges. *J Comp Physiol A* 164: 835–847.

FRANCO A (2012). *Caracterización ecológica de las comunidades intermareales rocosas a lo largo de una gradiente de productividad en las costas del Ecuador.* Disertación previa a la obtención del título de Licenciada en Ciencias Biológicas. Pontificia Universidad Católica del Ecuador. Quito, Ecuador.

GODDARD J H R (1991). Unusually large polar bodies in an aeolid nudibranch: A novel mechanism for producing extra-embryonic yolk reserves. *J Molluscan Stud* 57: 143–152.

GODDARD J H R (2004). Developmental mode in benthic opisthobranch molluscs from the northeast Pacific Ocean: Feeding in a sea of plenty. *Can J Zool* 82: 1954–1968.

GODDARD J and HERMOSILLO A (2008). Developmental mode in opisthobranch molluscs from the tropical eastern Pacific Ocean. *Veliger* 50: 83–96.

GODDARD J H R and GREEN B (2013). Developmental mode in Opisthobranch molluscs from the Northeast Pacific Ocean: Additional species from Southern California and supplemental data. *Bull S Calif Acad Sci* 112: 49–62.

GOSLINER T M (1995). The genus *Thuridilla* (Opisthobranchia: Elysiidae) from the tropical Indo-Pacific, with a revision of the phylogeny of the Elysiidae. *Proc Calif Acad Sci* 49: 1–54.

GOULDING M (2003). Cell contact-dependent positioning of the D cleavage plane restricts eye development in the *Ilyanassa* embryo. *Development* 130: 1181–1191.

GREENE R W (1970). Symbiosis in sacoglossan opisthobranchs: symbiosis with algal chloroplasts. *Malacologia* 10:357–368.

HADFIELD M G (1964). Opisthobranchia. The veliger larvae of the Nudibranchia. In *Fisches d'identification du zooplancton.* (Redige par Fraser, J. H., and B. J. Muus.) Conseil Permanent International pour l'Exploration de la Mer, Sheet No. 106, pp. 1–4.

HADFIELD M and SWITZER-DUNLAP M (1984). Opisthobranchs. In *The Mollusca* (Tompa AS, Verdonk NH, Van den Biggelaar JAM, eds.) London: Academic Press, 209–350.

HAGERMAN L (1970). The influence of low salinity on survival and spawning of *Elysia viridis* (Montagu) (Opisthobranchia, Sacoglossa). *Sarsia* 42: 1–6.

HAMATANI I (1960). Notes on veligers of Japanese opisthobranchs. *Publ Seto Mar Biol Lab* 8:59–70.

HANDELER K, GRZYMBOWSKI Y, KRUG P and WAGELE H (2009). Functional chloroplasts in metazoan cells: A unique evolutionary strategy in animal life. *Front Zool* 6:28

HENRY J and MARTINADALE M (1997), Nemerteans, the ribbon worms. In: Gilbert SF, Raunio *AM (eds) Embryology.* Constructing the organism. Sinauer, New York, pp 151–166

HEYLAND A, VUE Z, VOOLSTRA C R, MEDINA M and MOROZ L L (2011). Developmental transcriptome of *Aplysia californica.* J *Exp Zool Part B* 316: 113–134.

HOAGLAND K E, HOAGLAND K E, ROBERTSON R and ROBERTSON R (1988). An assessment of poecilogony in marine invertebrates: phenomenon or fantasy. *Biol Bull* 174: 109–125.

HOFFMAN C (1877). Beitrage zur Kenntnis der Nemertinen. I. Zur Entwicklungsgeschichte von *Tetrastemma varicolor* Oerst. *Niederl Arch Zool* 3: 205–215

HORWITZ R, JACKSON M D and MILLS S C (2017). The embryonic life history of the tropical sea hare *Stylocheilus striatus* (Gastropoda: Opisthobranchia) under ambient and elevated ocean temperatures. *PeerJ* 5: e2956.

INSTITUTO GEOGRÁFICO MILITAR (IGM). (1974). Mapa geológico del Ecuador: SANTA ELENA. Cartas topográficas. Available at: http://www.geoportaligm.gob.ec/portal/index.php/cartografia-de-libre-acceso-escala-50k/.

INSTITUTO GEOGRÁFICO MILITAR (IGM). (1974). Mapa geológico del Ecuador: JAMA. Cartas topográficas. Available at: http://www.geoportaligm.gob.ec/portal/index.php/cartografia-de-libre-acceso-escala-50k/.

KNOTT K E and MCHUGH D (2012). Introduction to symposium: Poecilogony - A window on larval evolutionary transitions in marine invertebrates. *Integr Comp Biol* 52: 120–127.

KRUG P J (2007). Poecilogony and larval ecology in the gastropod genus Alderia*. *Am Malacol Bull* 23: 99–111.

KRUG P J (2009). Not my "type": larval dispersal dimorphisms and bet-hedging in Opisthobranch life histories. *Biol Bull* 216: 355–372.

KRUG P J, GORDON D and ROMERO M R (2012). Seasonal polyphenism in larval type: Rearing environment influences the development mode expressed by adults in the sea slug *Alderia willowi. Integr Comp Biol* 52: 161–172.

KRUG P J, VENDETTI J E, ELLINGSON R A, TROWBRIDGE C D, HIRANO Y M, TRATHEN D Y, RODRIGUEZ A K, SWENNEN C, WILSON N G, VALDÉS A. A. (2015). Species selection favors dispersive life histories in sea slugs, but higher per-offspring investment drives shifts to short-lived larvae. *Syst Biol* 64: 983–999.

KRUG P J, VENDETTI J E and VALDÉS Á (2016). Molecular and morphological systematics of *Elysia* Risso, 1818 (Heterobranchia: Sacoglossa) from the Caribbean region. *Zootaxa* 4148: 1-137.

LANFEAR R, CALCOTT B, HO SYW and GUINDON S (2012) PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Mol Biol Evol* 29: 1695– 1701.

LEE C, KAANG B and LEE Y (2014). Spawning behavior and egg development of *Aplysia kurodai* inhabiting the coastal waters of Jeju Island, Korea. *Dev Reprod* 18: 25–31

LYONS D C, and HENRY J Q (2014). Ins and outs of spiralian gastrulation. *Int J Dev Biol* 58: 413–428.

MAcFARLAND F M (1924). XXV Expedition of the California Academy of Sciences to the Gulf of California in 1921. Opisthobranchiate Mollusca. *Proc Calif Acad Sci* 13: 389–420, pls. 10–21.

MACGINITIE G (1935). Ecological aspects of a California marine estuary. *Am Midl Nat* 16: 629-765; figs. 1-21 (ix-1935).

MADDISON W and MADDISON D (2015). Mesquite: a modular system for evolutionary analysis. Version 3.04. http://mesquiteproject.org/.

MARCUS E (1982). Systematics of the genera of the order Ascoglossa (Gastropoda). *J Moll Stud* 48 : 1–31.

MARTIN-DURAN J M and MARLETAZ F (2020). Unravelling spiral cleavage. *Development* 147: dev181081.

MARTÍNEZ-PITA I, SÁNCHEZ-ESPAÑA A I, and GARCÍA F J (2006). Some aspects of the reproductive biology of two Atlantic species of *Polycera* (Mollusca: Opisthobranchia). *J Mar Biolog Assoc* 86: 391–399.

MASLAKOVA S, MARTINDALE M, and NORENBURG J (2004). Fundamental proper-

ties of the spiralian developmental program are displayed by the basal nemertean *Carinoma tremaphoros* (Palaeonemertea, Nemertea). *Dev Biol* 267: 342–360

MCDONALD K, COLLIN R, and LESOWAY M (2014). Poecilogony in the Caenogastropod *Calyptraea lichen* (Mollusca: Gastropoda). *Invertebr Biol* 133: 213–220.

MEDINA M and WALSH P J (2000). Molecular systematics of the order Anaspidea based on mitochondrial DNA sequences (12S, 16S, and CO1). *Mol Phylogenet Evol* 15: 41–58.

PALUMBI S (1996), Nucleic acids II: The polymerase chain reaction. In: Hillis, D., Moritz C and Mable B (Eds), Molecular systematics. Sunderland, Sinauer Associates, pp. 205–247.

PIERCE S, CURTIS N, MASSEY S, BASS A, KARL S and FINNEY C (2006). A morphological and molecular comparison between *Elysia crispata* and a new species of kleptoplastic sacoglossan sea slug (Gastropoda: Opisthobranchia) from the Florida Keys, USA. *Molluscan Res* 26: 23–38.

RABINOWITZ J and LAMBERT J (2010). Spiralian quartet developmental potential is regulated by specific localization elements that mediate asymmetric RNA segregation. *Development* 137: 4039–4049.

RAHAT M (1976). Direct development and symbiotic chloroplasts in *Elysia timida* (Mollusca: Opisthobranchia). *Isr J Zool* 25: 186–193.

RAMBAUT A, DRUMMOND A J, XIE D, BAELE G and SUCHARD M A (2018). Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Syst Biol* 67: 901–904

REID J D (1964). The reproduction of the sacoglossan opisthobranch *Elysia maoria. Proc Zool Soc Lond* 143: 365-393

REYES F (2014). *Evaluación de la abundancia y distribución espacio-temporal de Opistobranquios con relación al tipo de sustrato en la zona rocosa intermareal de Ayangue y la entrada provincia de Santa Elena* (undergraduate thesis). Universidad Estatal. Santa Elena, Ecuador.

RIVEST B R (1978). Development of the eolid nudibranch *Cuthona nana* (Alder and Hancock, 1842), and its relationship with a hydroid and hermit crab. *Biol Bull* 154: 157–175.

RONQUIST F, TESLENKO M, VAN DER MARK P, AYRES D L, DARLING A, HÖHNA S, LARGET B, LIU L, SUCHARD M A and HUELSENBECK J P (2012). MrBayes 3.2: Bayesian phylogenetic efficient inference and model choice across a large model space. *Syst Biol* 61: 539–542.

SAUNDERS A and POOLE M (1910). The development of *Aplysia punctata*. *J Cell Sci* 55: 497–539.

TAYLOR P and MCCAIN E R (2010). Poecilogony as a tool for understanding speciation: Early development of *Streblospio benedicti* and *Streblospio gynobranchiata* (Polychaeta: Spionidae). *Invertebr Reprod Dev* 52: 91–101.

THOMPSON T E (1960). The importance of the larval shell in the classification of the sacoglossa and the acoela (gastropoda opisthobranchia). *J Mollus Stu* 34: 233–238.

THOMPSON T (1967). Direct development in a nudibranch, *Cadlina laevis*, with a discussion of developmental processes in Opisthobranchia. *J Mar Biol Assoc UK* 47: 1-22

THOMPSON T and SALGHETTI-DRIOLI U (1984). Unusual features of the development of the sacoglossan *Elysia hopei* in the Mediterranean Sea. *J Mollus Stu* 50: 61–63.

URIBE R, NAKAMURA K, INDACOCHEA A, PACHECO A, HOOKER Y and SCHRÖDL M (2013). A review on the diversity and distribution of Opisthobranch gastropods from Peru, with the addition of three new records. *Spixiana* 36: 43-60.

VENDETTI J E, TROWBRIDGE C D and KRUG P J (2012). Poecilogony and population genetic structure in *Elysia pusilla* (Heterobranchia: Sacoglossa), and reproductive data for five sacoglossans that express dimorphisms in larval development. *Integr Comp Biol* 52: 138–150.

VAN DEN BIGGELAAR J and HASZPRUNAR G (1996). Cleavage patterns and mesentoblast formation in the Gastropoda: an evolutionary perspective. *Evolution*: 50: 1520.

VON DÖREHN J (2015). Nemertea. In: Wanninger A, editor. Evolutionary developmental biology of invertebrates, vol. 2., Lophotrochozoa (Spiralia)Wien: *Springer*, pp. 155–92.

WOLF M and YOUNG C (2012). Complete development of the Northeast Pacific Arminacean Nudibranch *Janolus fuscus. Biol Bull* 222: 137–149.

WILSON C (1900). The habits and early development of *Cerebratulus lacteus* (Verrill). A contribution to physicological morphology. *Q J Microsc Sci* 43:97–198.

ZWICKL D (2006). *Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion*. Ph.D. dissertation, University of Texas, Austin, 125 pp.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

Spiralian model systems
Jonathan Q. Henry
Int. J. Dev. Biol. (2014) 58: 389-401
https://doi.org/10.1387/ijdb.140127jh

Beyond the sea: Crepidula atrasolea as a spiralian model system
Jonathan Q. Henry, Maryna P. Lesoway, Kimberly J. Perry, C. Cornelia Osborne, Marty Shankland and Deirdre C. Lyons
Int. J. Dev. Biol. (2017) 61: 479-493
https://doi.org/10.1387/ijdb.170110jh

Spiral cleavages determine the left-right body plan by regulating Nodal pathway in monomorphic gastropods, Physa acuta
Masanori Abe, Hiromi Takahashi and Reiko Kuroda
Int. J. Dev. Biol. (2014) 58: 513-520
https://doi.org/10.1387/ijdb.140087rk

Sipuncula: an emerging model of spiralian development and evolution
Michael J. Boyleand Mary E. Rice
Int. J. Dev. Biol. (2014) 58: 485-499
https://doi.org/10.1387/ijdb.140095mb

Variation in spiralian development: insights from polychaetes
Elaine C. Seaver
Int. J. Dev. Biol. (2014) 58: 457-467
https://doi.org/10.1387/ijdb.140154es

Ins and outs of Spiralian gastrulation
Deirdre C. Lyons and Jonathan Q. Henry
Int. J. Dev. Biol. (2014) 58: 413-428
https://doi.org/10.1387/ijdb.140151dl

Brachyury, Tbx2/3 and sall expression during embryogenesis of the indirectly developing polychaete Hydroides elegans
Cesar Arenas-Mena
Int. J. Dev. Biol. (2013) 57: 73-83
https://doi.org/10.1387/ijdb.120056ca