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

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Running title: *Elysia diomedea* in Ecuador: identification & development

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Abbreviations used in this paper: 16S, large ribosomal subunit rRNA; AV, animal-vegetal; BSA, bovine serum albumin; CMA BIO, advanced microscopy center; COI, cytochrome oxidase subunit I; DAPI, 4',6-diamidino-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; PBS, phosphate-buffered saline; PBT, phosphate-buffered saline with Triton; PCR, polymerase chain reaction; SYM, symmetrical model

ABSTRACT

_Elysia diomedea_ varies 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 (1) individuals from the three locations belonged to _E. diomedea_ and (2) 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 mm$^2$ of egg ribbon, and compared the cleavage pattern of this species to other heterobranchs and spiralian. _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. lecithotrophic 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 reproductive and phylogenetic aspects (Krug et al., 2016).
*Elysia diomedea* 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 & 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 & Smith, 1973). Goddard & 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 45º 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, Platyhelminthes and Mollusca (Chávez et al. 2017; Martin-Duran & 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 & 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 & Haszprunar 1996). In addition, and interesting but rare case of reproductive plasticity —interspecific variation in the type of larvae produced (e.g. poecillogony)— 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* 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.

**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 & 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 (NH₄)₂SO₄, 3 mM MgCl₂, 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 & 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 $30 \times 10^6$ generations. Each run had four chains with a temperature of 0.1. For each analysis, the chain was sampled every 1000 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 5000 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; 12:12h 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 20ml petri dishes with sterile seawater with salinity of 30 ± 1 ppt; 12:12h 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.

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 Figure 1. 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. 2). 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. 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 mm² 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 (1a², 1b², 1c² and 1d²) near the animal pole, whereas the the second quartet (2a, 2b, 2c and 2d) and macromeres (2A, 2B, 2C and 2D) 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 (Fig. 3J black arrow; Fig. 4E white arrow), 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 (Figure 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 & 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 1976; Ellingson & Krug 2006; Krug et al. 2016) and with the description of Bertsch & 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 & 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 & 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 spiralian. 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 & Lambert 2010; Lyons & Henry 2014). Because micromere quartets show conserved ectodermal and ectomesodermal fates in larval domains across spiralian, 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 spiralian, including several species of nemerteans and sipunculids, have been reported to display enlarged cells in the animal micromere quartets (Hoffman 1877; Wilson 1900; Henry & 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 & 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. lecithotrophic and capsular metamorphic development), can serve as extra-embryonic food reserve (Chia 1971; Bridges 1975; Clark & 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 many species of Elysia, regardless their developmental modes (Krug et al. 2016; Thompson & 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 & 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). Some controversy remains whether embryos are capable of ingesting EZY, as suggested for several sacoglossan species (Clark & Goetzfried 1978; Clark & 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 & 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 poecilocony. 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 & 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.

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Table 1. Embryonic developmental stages of *Elysia diomedea* within the egg capsule under ambient temperature (18-20° C)

| 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 |
Figure 1. *Elysia diomedea.* (A) Ballenita’s specimen of *E. diomedea.* (B) *E. diomedea* egg ribbon laid on the alga *Padina* sp. Scale bar 1 cm.
Figure 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.
Figure 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 1a, 1b, 1c, 1d, 2a, 2b, 2c, 2d, whereas the large micromeres (2a, 2b, 2c, 2d) and macromeres (2A, 2B, 2C, 2D) remain uncleaved. (H and 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 is 25 μm.
Figure 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 and 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) and (H), whereas a normal confocal microscopy was used to generate (C-E) and (G). Scale bar 25 μm.
Figure 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).
APPENDIX

Appendix 1. Specimens sequenced for this study and sequences obtained from GenBank

| Study code | Species          | Locality*                              | GenBank Accession number       |
|------------|------------------|----------------------------------------|--------------------------------|
| LAM001     | Elysia diomedea  | Ballenita, Ecuador                     | this study                     |
| LAM002     | Elysia diomedea  | Ballenita, Ecuador                     | this study                     |
| LAM003     | Elysia diomedea  | Ballenita, Ecuador                     | this study                     |
| LAM004     | Elysia diomedea  | Ballenita, Ecuador                     | this study                     |
| LAM005     | Elysia diomedea  | Ballenita, Ecuador                     | this study                     |
| LAM006     | Elysia diomedea  | Cabayu, Ecuador                        | this study                     |
| LAM007     | Elysia diomedea  | Cabayu, Ecuador                        | this study                     |
| LAM008     | Elysia diomedea  | Mompiche, Ecuador                      | this study                     |
| LAM009     | Elysia diomedea  | Mompiche, Ecuador                      | this study                     |
| LAM010     | Elysia diomedea  | Mompiche, Ecuador                      | this study                     |
| LAM011     | Elysia diomedea  | Mompiche, Ecuador                      | this study                     |
| LAM012     | Elysia diomedea  | Mompiche, Ecuador                      | this study                     |
| LAM013     | Elysia diomedea  | Mompiche, Ecuador                      | this study                     |
| LAM014     | Elysia diomedea  | Mompiche, Ecuador                      | this study                     |
| LAM015     | Elysia diomedea  | Mompiche, Ecuador                      | this study                     |
| LAM016     | Elysia diomedea  | Mompiche, Ecuador                      | this study                     |
| LAM017     | Elysia diomedea  | Mompiche, Ecuador                      | this study                     |
| TDI        | Elysia diomedea  | Costa Rica                             | DQ480193                       |
| TDI1       | Elysia diomedea  | Costa Rica                             | -                              |
| TDI2       | Elysia diomedea  | Costa Rica                             | DQ480194                       |
| 07BLR03    | Elysia diomedea  | Los Ríos, Panamá                       | KM204228                       |
| 07Jap01    | Elysia abei      | Misaki Marine Biological Station, the University of Tokyo, Japan | JN819137                       |
| Ea703LI    | Elysia amakusana | Lizard Island, Australia                | EU140851                       |
| 10TAA4     | Elysia amakusana | Niigata, Japan                         | AB759025                       |
| 06Van01    | Elysia asbecki   | NA, Vanuatu                            | KM204200                       |
| BC3471     | Elysia asbecki   | NA, Vanuatu                            | -                              |
| 02Jap01    | Elysia atroviridis | Choshi, Japan                       | KM204223                       |
| ESE2       | Elysia atroviridis | Okinawa, Japan                       | DQ480163                       |
| ESE3       | Elysia atroviridis | Okinawa, Japan                       | -                              |
| ESE4       | Elysia atroviridis | Okinawa, Japan                       | -                              |
| 07Aus03    | Elysia australis | Coulom, Australia                      | JN819142                       |
| 09Tha01    | Elysia bangtawaensis | Bang Tawa, Thailand                  | KM204224                       |
| Code    | Species Name         | Location                          | Accession Numbers |
|---------|----------------------|-----------------------------------|-------------------|
| Eb779Sa | Elysia bennettiae    | NA, Samoa                         | EU140868          |
| EBE2    | Elysia bennettiae    | NA, Guam                          | DQ480183          |
| EBE3    | Elysia bennettiae    | NA, Guam                          | -                 |
| 04SSal02| Elysia buonoi        | Bahamas                           | JQ914621          |
| 10Dry01 | Elysia canguzua      | Dry Tortugas, United States       | KM204225          |
| ECA2    | Elysia canguzua      | Florida, United States            | -                 |
| ECA3    | Elysia canguzua      | Florida, United States            | DQ480196          |
| ECA4    | Elysia canguzua      | Florida, United States            | -                 |
| 09Tha0001| Elysia chilkensis    | NA, Singapore                     | KM204209          |
| 06Mas01 | Elysia chlorotica    | Martha's Vineyard, United States  | KM204226          |
| ECL     | Elysia chlorotica    | Massachusetts, United States      | -                 |
| ECL24   | Elysia chlorotica    | Massachusetts, United States      | DQ480200          |
| ECL4    | Elysia chlorotica    | Massachusetts, United States      | DQ471222          |
| 06Jam01 | Elysia cornigera     | Discovery Bay, Jamaica            | JN819125          |
| 06FL01  | Elysia crispata      | Summerland Key, United States     | JN819139          |
| ECR37   | Elysia crispata      | NA, Belize                        | DQ480199          |
| ECR42   | Elysia crispata      | ambiguous in literature           | -                 |
| ECR46   | Elysia crispata      | ambiguous in literature           | DQ480198          |
| 09Gua01 | Elysia degeneri      | Bile Bay, Guam                    | KM204227          |
| 10Npr01 | Elysia ellenae       | New Providence, Bahamas           | JN819141          |
| 06MUS01 | Elysia flava         | Bocas del Toro, Panamá            | KM204229          |
| 11How02 | Elysia furvacauda    | Lord Howe Island, Australia       | KM204218          |
| Eham02Jap01| Elysia hamatanii   | Misaki Marine Biological Station, the University of Tokyo, Japan | JN819143 | JN819110 |
| 05SD01  | Elysia hedgpethi     | San Diego, United States          | KM204230          |
| 09Tha001| Elysia leucolegnote  | Bang Tawa, Thailand               | KM204231          |
| 11Maui01| Elysia lobata        | Hekili Point (Hawaii, Maui), United States | KM204232 | KM086382 |
| Em726Su | Elysia macnaei       | NW-Sulawesi, Indonesia            | EU140854          |
| ZSM20033821| Elysia macnaei    | Sulawesi, Indonesia               | -                 |
|         |                      |                                   | GQ996689          |
| Code | Species              | Location                        | GenBank 1 | GenBank 2 |
|------|----------------------|---------------------------------|-----------|-----------|
| 06NZ01 | *Elysia maoria*      | Auckland (Waitemata Harbor), New Zealand | KM204233 | KM086383 |
| 06Jam001 | *Elysia marcus*      | Discovery Bay, Jamaica          | KM204234 | KM086384 |
| 09Gua01 | *Elysia mercieri*     | Bile Bay, Guam                 | -         | KM086385 |
| 10Jap01 | *Elysia minima*       | Chinen (Okinawa), Japan        | KM204235 | KM086386 |
| ENI1  | *Elysia nigrocapitata* | Okinawa, Japan                | DQ480175 | DQ471226 |
| ENI2  | *Elysia nigrocapitata* | Okinawa, Japan                | -         | DQ471227 |
| ENI3  | *Elysia nigrocapitata* | Okinawa, Japan                | -         | DQ471228 |
| 09Jap01 | *Elysia obtusa*       | Kominato, Japan                | KM204236 | KM086387 |
| EOB   | *Elysia obtusa*       | Okinawa, Japan                | DQ480182 | -         |
| EOB1  | *Elysia obtusa*       | Okinawa, Japan                | -         | DQ471259 |
| CPICC00842 | *Elysia orientalis*    | Florida, United States        | KP187839 | KP187842 |
| 06Jam0001 | *Elysia ornata*     | Discovery Bay, Jamaica        | JN819132 | JN819093 |
| EOR3  | *Elysia ornata*       | ambiguous in literature        | DQ480177 | -         |
| EOR4  | *Elysia ornata*       | ambiguous in literature        | DQ480178 | DQ471261 |
| EOR7  | *Elysia ornata*       | ambiguous in literature        | DQ480179 | DQ471262 |
| EOR8  | *Elysia ornata*       | ambiguous in literature        | DQ480180 | DQ471263 |
| EOR9  | *Elysia ornata*       | ambiguous in literature        | DQ480181 | -         |
| 04Swe04 | *Elysia papillosa*     | Sweetings Cay, Bahamas        | JN819145 | JN819108 |
| 06Tar01 | *Elysia papillosa*     | Florida, United States        | KP187840 | KP187843 |
| 07Pla04 | *Elysia pratensis*    | Plana Cays, Bahamas           | KM204237 | JN819112 |
| EPR   | *Elysia pratensis*    | NA, Bahamas                    | DQ480162 | -         |
| EPR1  | *Elysia pratensis*    | NA, Bahamas                    | -         | DQ471258 |
| 04Jap02 | *Elysia pusilla*      | NA, Japan                      | JN819152 | -         |
| 07Aus01 | *Elysia pusilla*      | Coulom, Australia             | JN819151 | -         |
| EPU11 | *Elysia pusilla*      | Hawaii, United States         | DQ480201 | -         |
| 0001  | *Elysia pusilla*      | NA, Australia                 | -         | JQ914593 |
| 00014 | *Elysia pusilla*      | NA, Japan                      | -         | JQ914606 |
| 08Jap01 | *Elysia rufescens*   | Sobe (Okinawa), Japan         | KM204247 | KS573688 |
| ERU1  | *Elysia rufescens*    | ambiguous in literature        | DQ480171 | DQ471237 |
| ERU6  | *Elysia rufescens*    | ambiguous in literature        | DQ480172 | DQ471238 |
| Identifier | Species                | Location/Specific Details                        | Accession Numbers |
|------------|------------------------|--------------------------------------------------|-------------------|
| 06Lee03    | Elysia serca           | Lee Stocking Island, Bahamas                      | KM204248          |
| ESR2       | Elysia serca           | NA, Bahamas                                       | DQ471242          |
| ESR3       | Elysia serca           | NA, Bahamas                                       | DQ480192 DQ471243 |
| ESR4       | Elysia serca           | NA, Bahamas                                       | DQ471244          |
| 09Tha02    | Elysia singaporensis   | Western Johor Strait, Singapore                   | KM204249          |
| 07Aus001   | Elysia stylifera       | Point Cartright, Australia                        | GU191046 -        |
| 06Jam03    | Elysia subornata       | Discovery Bay, Jamaica                            | JN819135 JN819111 |
| ESR2       | Elysia subornata       | Florida, United States                            | DQ480165 DQ471283 |
| 05Jap01    | Elysia sugashimae      | Sobe (Okinawa), Japan                             | KM204251 KM086399 |
| 10Jap001   | Elysia thompsoni       | Chinen (Okinawa), Japan                           | KM204252 JN819088 |
| ETU13      | Elysia trisinuata      | Lizard Island, Australia                          | DQ480186          |
| Etri02Jap01| Elysia trisinuata      | Hayama, Japan                                     | KM204253 KM086401 |
| ETU2       | Elysia velutinus       | ambiguous in literature                           | DQ480184 DQ471249 |
| ETU8       | Elysia velutinus       | ambiguous in literature                           | DQ480185 DQ471251 |
| ETU25      | Elysia velutinus       | ambiguous in literature                           | - DQ471250        |
| 05Ire01    | Elysia viridis         | Galway, Ireland                                   | KM204254 KM086403 |
| EVI        | Elysia viridis         | NA, Portugal                                      | DQ480195 -        |
| EVI3       | Elysia viridis         | NA, Portugal                                      | - DQ471211        |
| 07Swe01    | Elysia zuleicae        | Sweetings Cay, Bahamas                            | JN819146 JN819105 |
| 01Jap04    | Plakobranchus sp.      | Okinawa, Japan                                    | - KC573714        |
| 04Jap01    | Plakobranchus sp.      | Okinawa, Japan                                    | KM204278 KC573718 |
| 09Gua06    | Plakobranchus sp.      | Bile Bay, Guam                                    | KM204279 -        |

* City/Specific location and country where it was possible, just country, or not information available (NA). Ambiguous in literature means that information included different locations.