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Embryonic development and implication for colonization of three ascidian species with different biogeographic status in Patagonia, Argentina

Desarrollo embrionario e implicaciones para la colonización de tres especies de ascidias con diferente estatus biogeográfico en la Patagonia, Argentina

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ABSTRACT

Background. Ascidians (Chordata, Tunicata) have a free-swimming larva that develops into a sessile adult. Solitary species generally have external fertilization, although some perform internal fertilization, incubating the larvae before releasing them into water. These reproductive modes influence colonization success by affecting development time, exposure to environmental factors, and predation. **Goals.** This study compared the embryonic development of three solitary ascidians species from the intertidal zone of Río Negro, Argentina, with different biogeographic status in the area: *Ciona robusta* (invasive), *Paramolgula gregaria* (native) and *Asterocarpa humilis* (cryptogenic; i.e., when the available evidence is insufficient to determine the species' origin). **Methods.** Eggs and sperm were extracted from individuals by dissection. Eggs were hydrated and fertilized, except in *A. humilis*, where cross-fertilization was unsuccessful, and embryos at different stages of development were collected from an incubating individual. Embryonic development, from embryo to larva, was recorded for all three species, including larval size measurements. **Results.** Ten developmental stages were identified in *P. gregaria*, and eight stages in both *A. humilis* and *C. robusta*, with all species completing their cycles in an urodele larval form. *Ciona robusta* showed the shortest overall development time, *P. gregaria* displayed the highest proportion of embryos successfully reaching the motile larval stage, while *A. humilis* exhibited the largest larva among the studied species. **Conclusions.** The differences in development time, larval size, and success rates suggest distinct adaptive strategies in each species, potentially influencing their establishment and dispersal capacities. This study provides novel data on the embryonic development of *Paramolgula* and *Asterocarpa*, shedding light on reproductive mechanisms that support the colonization and persistence of species with different biogeographic statuses in the Río Negro intertidal zone.

Key words: Fertilization, invasive species, larva, reproductive success, tunicates

RESUMEN

Antecedentes. Las ascidias (Chordata, Tunicata) tienen una larva de vida libre que se desarrolla en un adulto sésil. Las especies solitarias generalmente presentan fecundación externa, aunque algunas realizan fecundación interna, incubando larvas antes de liberarlas al agua. Estos modos reproductivos influyen en la colonización al afectar el desarrollo, la exposición a factores ambientales y la depredación. **Objetivos**. Comparar el desarrollo embrionario de tres especies de ascidias solitarias de la zona intermareal de Río Negro, Argentina, con distinto estatus biogeográfico: *Ciona robusta* (invasora), *Paramolgula gregaria* (nativa) y *Asterocarpa humilis* (criptogénica). **Métodos**. Se extrajeron óvulos y espermatozoides mediante disección. Los óvulos fueron hidratados y fecundados, excepto en *A. humilis*, donde la fecundación cruzada no fue exitosa y se recolectaron embriones en distintos estadios a partir de un individuo incubador. Se registró el desarrollo embrionario hasta la fase larval y se midió el tamaño de las larvas. **Resultados.** Se identificaron 10 estadios de desarrollo en *P. gregaria* y ocho estadios tanto en *A. humilis* como en *C. robusta*, completando todas las especies sus ciclos en una forma larval de tipo urodelada. *Ciona robusta* mostró el menor tiempo total de

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desarrollo, *P. gregaria* exhibió la mayor proporción de embriones que alcanzaron exitosamente la etapa larval móvil, mientras que *A. humilis* presentó las larvas de mayor tamaño entre las especies estudiadas. **Conclusiones.** Las diferencias en tiempo de desarrollo, éxito reproductivo y tamaño larval sugieren estrategias distintas, influyendo en el establecimiento y dispersión de cada especie. Este estudio aporta datos inéditos sobre el desarrollo embrionario de *Paramolgula y Asterocarpa*, revelando mecanismos reproductivos que favorecen la colonización de especies con distinto estatus biogeográfico en la zona intermareal de Río Negro.

Palabras clave: Especie invasora, éxito reproductivo, fertilización, larva, tunicados

INTRODUCTION

Invasive species represent one of the most significant threats to global biodiversity, driving ecosystem transformations and disrupting ecological balances (Reyna *et al.*, 2023). Among marine taxa, invasive ascidians are particularly impactful in benthic communities, where they can dominate space, alter trophic interactions, reduce native biodiversity, and affect aquaculture operations (Rius *et al.*, 2009; Zhan *et al.*, 2015). Ascidians are frequent marine invaders due to their rapid growth, efficient reproduction, and tolerance to environmental stressors, including salinity, temperature fluctuations, and pollution (Giachetti *et al.*, 2022). These traits, combined with their ability to colonize artificial habitats such as marinas and aquaculture facilities, position ascidians as a globally relevant group for the study of marine invasions.

Understanding the mechanisms that underlie the establishment and dispersal of invasive ascidians is essential for managing their impacts. Although the ecological and physiological traits of adult ascidians have been extensively studied, the early stages of their life cycle —embryogenesis, larval development, and settlement— remain underexplored (Pineda *et al.*, 2012). These stages are crucial, as they determine the viability of populations and their ability to adapt to new environments (Hoyer *et al.*, 2024; Rosner & Rinkevich, 2024). Investigating embryonic development provides valuable insights into species-specific responses to abiotic factors, reproductive strategies, and the evolutionary mechanisms that may drive invasion success (Rosner & Rinkevich, 2024).

Ascidians are an ideal model for studying embryonic development, due to their phylogenetic position as the closest invertebrate relatives to vertebrates, their simple body plan, and their highly conserved mosaic developmental strategy. These organisms exhibit a simple and efficient life cycle with distinct larval and adult stages, making them also a valuable model for studies in ecology and evolutionary processes (Jeffery, 1994; Lemaire, 2011). The embryonic development of ascidians is characterized by highly stereotypical cleavage patterns and a mosaic developmental program, offering insights into chordate body plan formation (Conklin, 1905; Nishida & Satoh, 1983; Hotta *et al.*, 2007; Funakoshi *et al.*, 2021).

The development of staging tables, particularly for species like *Ciona intestinalis* (Linnaeus, 1767), *Phallusia mammillata* (Cuvier, 1815), and *Halocynthia roretzi* (Drasche, 1884), has facilitated comparative studies across ascidian species and established baselines for developmental timing and morphology (Hotta *et al.*, 2007; Lemaire, 2009; Funakoshi *et al.*, 2021). However, differences in staging criteria and nomenclature among studies have highlighted the need for standardized frameworks to compare developmental stages across species (Hotta *et al.*, 2020).

Unlike *C. intestinalis*, whose embryogenesis has been mapped in extraordinary detail (Conklin, 1905; Nishida, 1987; Jeffery, 1992; Satoh, 1994; Rhee *et al.*, 2005; Munro *et al.*, 2006; Hotta *et al.*, 2007; Clutton *et al.*, 2021), the timing, morphological characteristics, and functional adaptations of embryonic development remain poorly understood in many other species. The study of new species highlights the need to diversify research beyond well-established model organisms, such as *C. intestinalis*. Investigating species with unique traits not only enhances our understanding of ascidian biology but also provides insights into the evolutionary plasticity of chordates. Such studies are crucial for reconstructing the ancestral traits of chordates and understanding how developmental and ecological pressures shape body plan adaptations.

Comparative embryological studies are also quite important in understanding the roles of early life stages in the establishment of invasive species. For instance, variations in developmental timing, larval morphology, or environmental sensitivity could influence dispersal potential, settlement success, and competitive interactions. This is especially relevant in locations like Playas Doradas, Río Negro, Argentina, where ascidian communities are composed of species with distinct biogeographic status: the invasive *Ciona robusta* Hoshino & Tokioka, 1967, the native *Paramolgula gregaria* (Lesson, 1830) and the cryptogenic *Asterocarpa humilis* (Heller, 1878). Comparative analyses of embryonic development in these species can reveal interspecific differences that may explain their contrasting patterns of establishment and dispersal in this coastal area.

Ciona robusta is a highly invasive species with a global distribution facilitated by maritime activities (Schwindt *et al.*, 2020; Shenkar *et al.*, 2024). Originally native to the northwestern Pacific, it has colonized temperate coastal regions worldwide, thriving in artificial habitats (Robinson *et al.*, 2017; Wilson *et al.*, 2022). Its rapid growth and broad environmental tolerance make it a successful invader (Giachetti *et al.*, 2022; Wilson *et al.*, 2022).

Paramolgula gregaria is a native species found primarily along the coasts of southern South America, including Argentina and Chile, where it inhabits stable subtidal environments (Lagger *et al.*, 2009). *Paramolgula gregaria* has not been reported in non-native regions. This species, a member of the order Stolidobranchia, represents a lineage that diverges significantly from Phlebobranchia like *Ciona*. Notably, some Stolidobranchia are known for their truncated larval stages, including tailless larvae, which reflect a departure from the chordate archetype (Huber *et al.*, 2000).

On the other hand, *Asterocarpa humilis*, also a member of Stolidobranchia, represents an intriguing case due to its cryptogenic status (Carlton, 1996; Schwindt *et al.*, 2020). It has been recorded in various regions, including the southern hemisphere's temperate coasts, such as New Zealand, South America, and parts of Australia, as well as in regions where its presence may be linked to anthropogenic activities (Taverna *et al.*, 2021; Shenkar *et al.*, 2024). Its uncertain biogeographic origins and increasing presence in various marine ecosystems from the northern hemisphere (Bishop *et al.*, 2012; Nall *et al.*, 2015), have raised questions about its potential as an invasive species (Taverna *et al.*, 2021).

This study aims to describe and compare the embryonic development of three solitary ascidian species from Playas Doradas (Rio Negro Province, Argentina), from fertilization to the larval stage, to establish a detailed developmental framework for each species. This approach seeks to uncover developmental traits that may contribute to the comprehension of their biology and ecological success. Through this study, we seek to contribute to a deeper understanding of the mechanisms driving establishment and dispersal processes in both invasive and native ascidians, thus providing a basis for future research and management strategies.

MATERIALS AND METHODS

The study was conducted from 6 to 10 November 2017 at Playas Doradas, a beach located in San Matías Gulf, Río Negro Province, Argentina (41°37′52″S, 65°01′45″W) (Fig. 1A). The area features a rocky intertidal zone primarily composed of deposits of limestone, sandy limestone, partly dolomitic and coquina, of a grayish white color, sometimes dark gray due to the effects of weathering, belonging to El Fuerte Formation (Busteros *et al.*, 1998), with a gradual slope towards the sea. Specimens were collected during low tide from the shallow intertidal zone, beneath rocks in flooded areas. While specimens of *Ciona robusta* (n=5; Fig. 1B) and *Asterocarpa humilis* (n=5; Fig. 1C) were collected attached to rocks, those of *Paramolgula gregaria* (n=5; Fig. 1D) were loose from the subtidal after high tides. *In situ* water temperature was recorded at 17°C.

Gamete extraction followed the protocol outlined by Lambert (2014). To prevent the unintended release of gametes and to encourage their accumulation, the specimens were kept under continuous light throughout the process. Before beginning extraction, epibionts and debris were carefully removed from the tunic of the ascidians. To isolate the specimens, the tunics were removed by making an incision between the siphons. Once the tunics were removed, the specimens were placed in a Petri dish containing filtered seawater. Due to anatomical differences, the gamete extraction process varied depending on the order of the specimens. Sperm was collected by making an incision at the end of the sperm duct and using a Pasteur pipette to aspirate the content. Oocytes were collected by making a small incision in the ovarian region and gently sliding a Pasteur pipette so the oocytes adhered to its exterior.

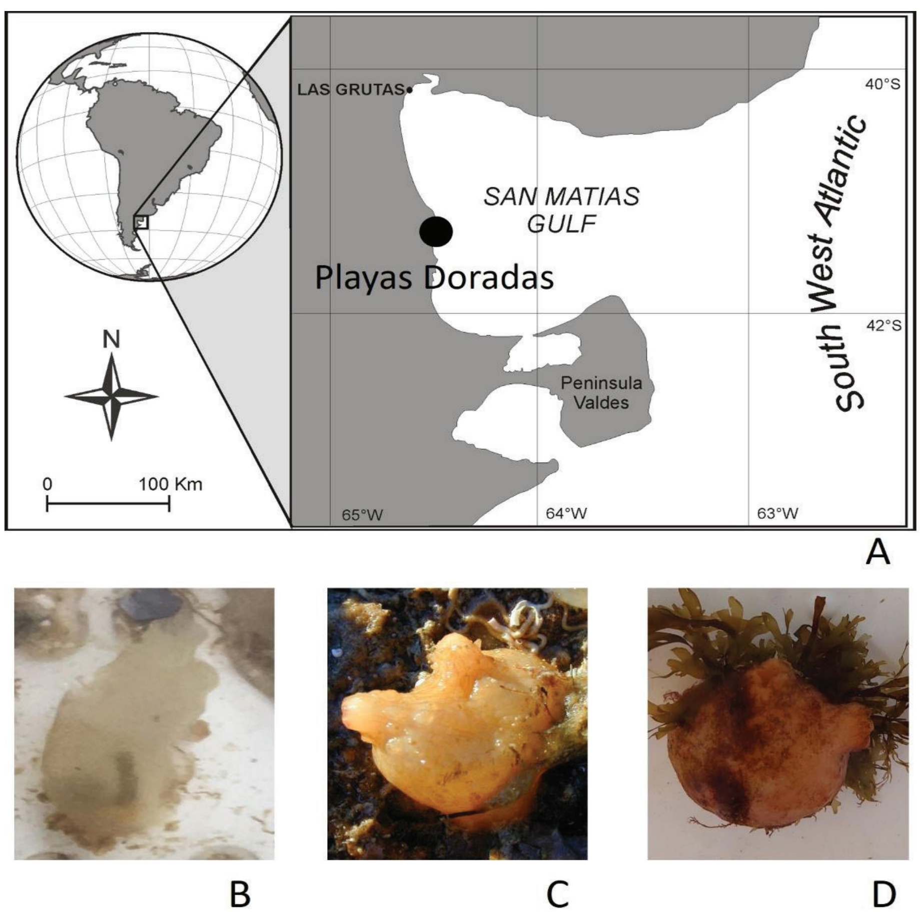

Figure 1. A. Location of the study area (adapted from Lagger & Tatián, 2013). B. Specimen of *Ciona robusta*. C. Specimen of *Asterocarpa humilis*. D. Specimen of *Paramolgula gregaria*.

In Stolidobranchia, such as *P. gregaria*, the gonads are located beneath the branchial basket. After isolating the animal, the branchial basket was removed to access the gonads. For both oocytes and sperm, a Pasteur pipette was inserted into the ovary and testis (or sperm duct in the case of *C. robusta*), respectively, and the contents were aspirated. Oocytes from each individual were placed in filtered seawater in separate beakers and repeatedly washed using a 100 µm pore-size filter. The filter removed immature oocytes, which, due to their smaller size, passed through the pores while retaining the mature ones. Sperm from each individual was collected in separate individual plastic tubes (2 ml) and kept refrigerated until fertilization. Before fertilization, the viability of the sperm was checked by observing motility under a light microscope. For cross-fertilization, sperm was diluted in seawater and mixed in a beaker with oocytes from another individual of the same species.

In *C. robusta*, sperm from specimen 4 was mixed with oocytes from specimens 1 and 5. For *P. gregaria*, two fertilizations were performed. In the first, sperm from specimen 1 was mixed with oocytes from specimens 2 and 3. In the second, sperm from specimens 2 and 3 was combined with oocytes from specimen 4. In the case of *A. humilis*, since cross-fertilization was not achieved, embryos at various developmental stages were collected from a specimen that was incubating them at that time.

Fertilized oocytes were maintained at 18°C until the experiment concluded. This value is close to the average range of temperatures measured in intertidal tide pools in a nearby area (Islote Lobos, on the west coast of the San Matías Gulf) year-round, between October 2005 and September 2007 (Ocampo Reinaldo & Storero, 2007).

To assess reproductive performance, we estimated three key parameters commonly used in marine invertebrate research: (a) fertilization success, defined as the percentage of oocytes that were successfully fertilized; (b) reproductive success, calculated as the percentage of fertilized oocytes that reached the motile larval stage; and (c) development time, measured from the first embryonic stage to the appearance of motile larvae. These parameters provide a standardized framework to compare reproductive efficiency and developmental rates among species, following approaches previously applied in ascidian studies (Jantzen *et al.*, 2001; Phillippi *et al.*, 2004).

Developmental stages were identified, measured, and counted using a counting chamber under a light microscope. Observations were made at 1 h intervals for *C. robusta* and 2 h intervals for *P. gregaria*. Embryo diameter was measured from digital micrographs using ImageJ calibrated with a stage micrometer. Although oocyte diameter was not directly measured before fertilization, embryo size at early stages serves as a reliable proxy for initial egg size in ascidians, as fertilization typically occurs immediately after spawning or dissection.

RESULTS

Fertilization success reached 75% in *Ciona robusta* and 70% in *Paramolgula gregaria*. Reproductive success was 2.28% in *C. robusta* (n = 1,487 oocytes) and 11.58% in *P. gregaria* (n = 1,079 oocytes). The development time was 5 h in *C. robusta* and 18 h in *P. gregaria*. The eggs of the three studied species presented the typical structure with a chorion enclosing the perivitelline fluid, and outer follicle cells adhering to the surface of the chorion. However, they showed considerable variation in the size and shape of the perivitelline structures (Figs. 2A, 3A, 4A). In *A. humilis*, whose cross-fertilization was not achieved, and *P. gregaria*, inner follicular cells were observed floating freely within the follicular space, displaying different arrangements in each species (Figs. 3B, 4A-C). In *P. gregaria*, these cells appeared flattened against the egg membrane, while outer follicular cells were scarce and barely distinguishable. In contrast, the outer follicular cells in *Ciona robusta* showed a radiated shape due to their characteristic enlargement (Fig. 2B-H). Finally, in *C. robusta* and *P. gregaria,* the ovum appears somewhat in an eccentric position, whereas in *A. humilis* a lifting of the chorion is observed with a lateral movement of the ovum (Figs. 2A-B, 3A-B, 4A-B).

We recognized several of the discrete 26 developmental stages described so far for ascidians in the three species analyzed, from eggs through the hatching larvae, on the basis of readily discernible and unambiguous external morphological features. Stage 1 spans from fertilization to the completion of the first mitotic division. The embryo is a single cell approximately 200 µm in diameter in the three species, containing a uniform cytoplasm (Figs. 2A, 3A, 4A-B). At this stage, cytoplasmic rearrangements were initiated, setting the basis for later development. The first mitotic spindle was positioned along the animal-vegetal axis, with two daughter cells visible but not fully separated.

During the cleavage period (Figs. 2B-C, 3C-D, 4C-D), the second mitotic division separated the embryo into anterior and posterior halves. Initially, the four blastomeres were loosely associated, but compaction toward the end of the stage shaped a more cohesive spherical embryo. Blastomeres showed slightly different sizes, and compaction occurred more rapidly in *C. robusta*, indicating subtle differences in the timing of morphogenetic processes.

At stage 8 (Figs. 2D, 3E, 4E), the embryos contained 64 cells arranged in a characteristic square-like pattern when viewed from the animal pole. Vegetal blastomeres were visibly prominent due to their bulging shape, which contributes to the flattening of the vegetal side in preparation for gastrulation. However, the larger overall size and differences in cleavage symmetry between the three species were notable (Figs. 2E, 3E-G, 4E-F).

Stage 14 marked the onset of neurulation (Figs. 2F, 3H, 4G). The embryos exhibited an oval-shaped morphology with a neural plate forming a shallow furrow. This furrow will eventually develop into the neural tube. Neural tube zippering began posteriorly and proceeded anteriorly during this period. In *P. gregaria,* the embryo exhibited a diamond-shaped morphology.

At stage 20 (Figs. 2G-H, 3I, 4H), during the early tail bud period, *C. robusta* and *P. gregaria* embryos demonstrated a conspicuous tail bend, marking complete neuropore closure and the end of neurulation. The tail length became approximately equal to the trunk length, representing an intermediate stage in body plan development. The embryos began to lengthen. In both species, stage 22 (Figs. 2I-J, 3J) represented the transition to a fully circularly curved body, with the tail bending into a half-circle. The tail length was approximately twice that of the trunk, and exhibited the large cells of the notochord.

By stage 26, larvae hatched (Figs. 2K, 3K, 4I). The tail, which had been curved and touching the trunk during the embryonic stages, straightened. The notochord appeared as a uniform tubular structure that extended from the posterior region of the head to the tip of the tail. The

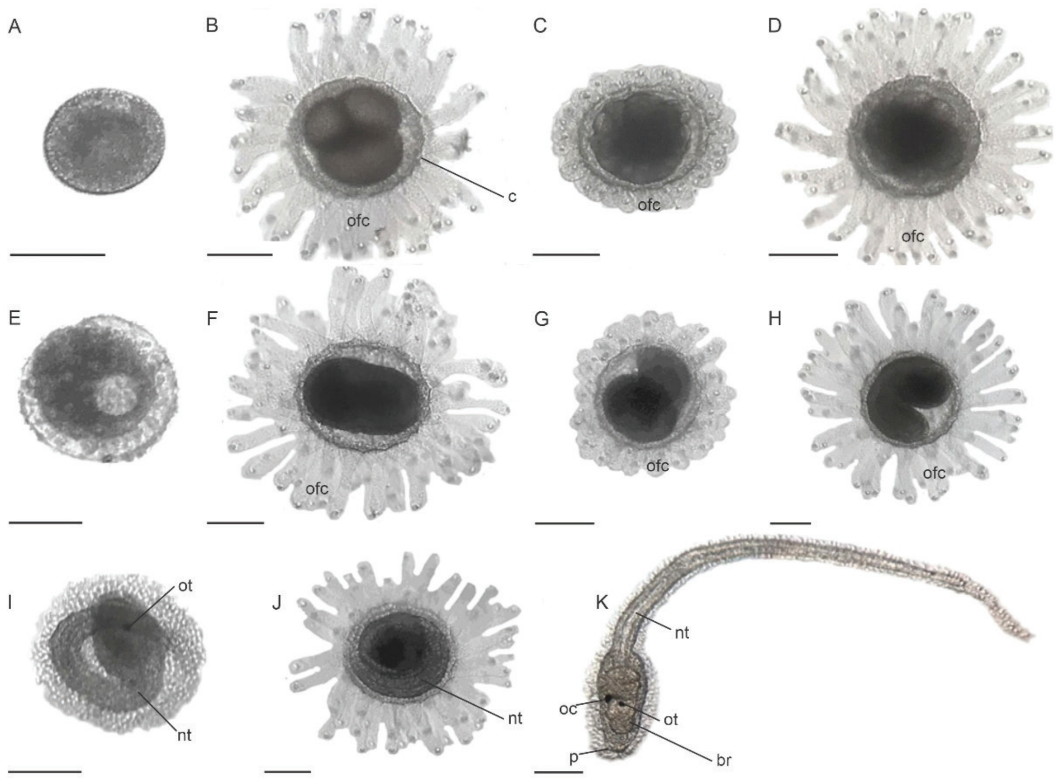

Figure 2. External morphology of developing embryos of *Ciona robusta*. Stages of Hotta *et al*. (2007) are indicated. A. Egg. B. Stage 3: Four-cell embryo. C. Stage 5: All animal and vegetal cells have undergone a fourth cleavage. D. Stage 8: The embryo has an almost circular shape. E. Stage 10: Gastrulation begins. F. Stage 14: Early neurula. G. Stage 19: Early tailbud period in which the tail differentiates. H. Stage 20: The tail bends and the neurulation ends. I. Stage 23: The body curves in a circular shape and the pigmentation of the otolith begins. The notochord is evident. J. Stage 24: The tail strongly curves, with the tip surpassing the anterior end of the trunk. K. Hatching larva. Abbreviations: br= brain; c= chorion; nt= notochord; oc= ocellus; ofc= outer follicular cells; ot= otolith; p= papillae. Scale bar= 100 μm.

trunk had an elongated rectangular shape and varied in size between the three species, being larger in *P. gregaria* and almost similar in the other species. Additionally, the proportion of the trunk to body length varied, being larger in *P. gregaria* as well. Therefore, this species showed a smaller tail than the other species. In the anterior trunk of *A. humilis*, three apical adhesive papillae were discernible.

The estimated rate of increase of the egg size across different stages varied between species. In *C. robusta*, there was a significant increase at the beginning of development, followed by a second increase at the end of the cleavage period. In *A. humilis*, egg size remained virtually unchanged throughout development. In contrast, in *P. gregaria*, the size increase began during the neurula period (Fig. 5A). Despite these variations, the embryo size did not differ among the three species, remaining very similar until the middle of the tailbud period. At this point, pronounced growth rates led to differences in larval size, with *A. humilis* producing the largest larvae (1.383 ± 0.145 mm); *C. robusta* the intermediate (1.314 ± 0.076) and *P. gregaria* the smallest (1.105 ± 0.185) (Fig. 5B). Figure 5C depicts the delayed development of *P. gregaria* in comparison with that of *C. robusta*.

DISCUSSION

The comparative embryological analysis of *C. robusta*, *P. gregaria*, and *A. humilis* reveals variations in fertilization and reproductive success, development time, growth, and morphological traits. These findings provide insights into the mechanisms underlying the establishment, dispersal, and invasive potential of these ascidian species and emphasize the importance of understanding their embryological development for both evolutionary and ecological studies.

In *C. robusta* and *P. gregaria*, the fertilization of eggs was achieved and the fertilization success varied slightly, reaching a higher percentage in *C. robusta*. Fertilization success in ascidians varies significantly among species due to differences in reproductive strategies, gamete characteristics, and environmental sensitivity. In *Pyura praeputialis* (Heller, 1878), for instance, fertilization efficiency is closely linked to gamete quality and timing, with both sperm and eggs showing reduced viability over time, highlighting the importance of synchronized spawning and favorable environmental conditions (Manríquez & Castilla, 2010). In contrast, species like *Ascidia mentula* Müller, 1776 store gametes in gonoducts, allowing for extended fertilization windows, though success remains influenced by external factors such as water movement and temperature (Havenhand, 1991). Egg size and morphology also play a key role: in *Styela plicata* (Lesueur, 1823), eggs with a larger area of follicular cells exhibit higher fertilization rates across a range of sperm concentrations, suggesting that structural features of the egg can improve gamete encounter rates and compatibility (Crean & Marshall, 2015).

The initial egg size differed among the three species, being larger in *A. humilis* and smaller in *C. robusta*. Egg size is a key determinant of the energy reserves available to developing larvae, with larger eggs providing more substantial yolk content for nourishment. In lecitotrophic larvae, such as those of ascidians, the energy provided by the yolk is crucial for development until the larva settles or undergoes metamorphosis. Larger eggs are typically associated with slower development, as they support longer periods of yolk dependence, but may also contribute to enhanced larval survival in environments with limited external resources (Havenhand, 1995). On the other hand, smaller eggs, as seen in *C. robusta*, may promote more rapid development, leading to earlier settlement and a potentially broader dispersal range. This trade-off between egg size and development rate has significant implications for reproductive success and the ecological distribution of ascidians, particularly concerning their invasive potential. The smaller egg size of *C. robusta*, combined with its faster development, may enhance its ability to rapidly establish and spread in new environments, a key trait for its success as an invasive species (Lambert, 2005).

Compared to the literature, subtle yet biologically meaningful differences in the timing and morphology of embryos were observed (Hotta *et al*., 2007; Lemaire, 2009; Funakoshi *et al*., 2021). For instance, while the cleavage period in *C. robusta* progresses with relatively consistent symmetry, *P. gregaria* exhibits asynchronous divisions in vegetal blastomeres, potentially reflecting adaptations to environmental conditions. Similar heterochronies or alterations in cleavage patterns have been interpreted as responses to environmental constraints such as temperature, oxygen availability, or salinity in other marine invertebrates (Przeslawski, 2004; Byrne, 2011). For example, in echinoderms and polychaetes, delayed or asynchronous cleavage has been linked to adaptation to cold or variable environments, as slower or desynchronized development may enhance tolerance to stress or allow better syn-

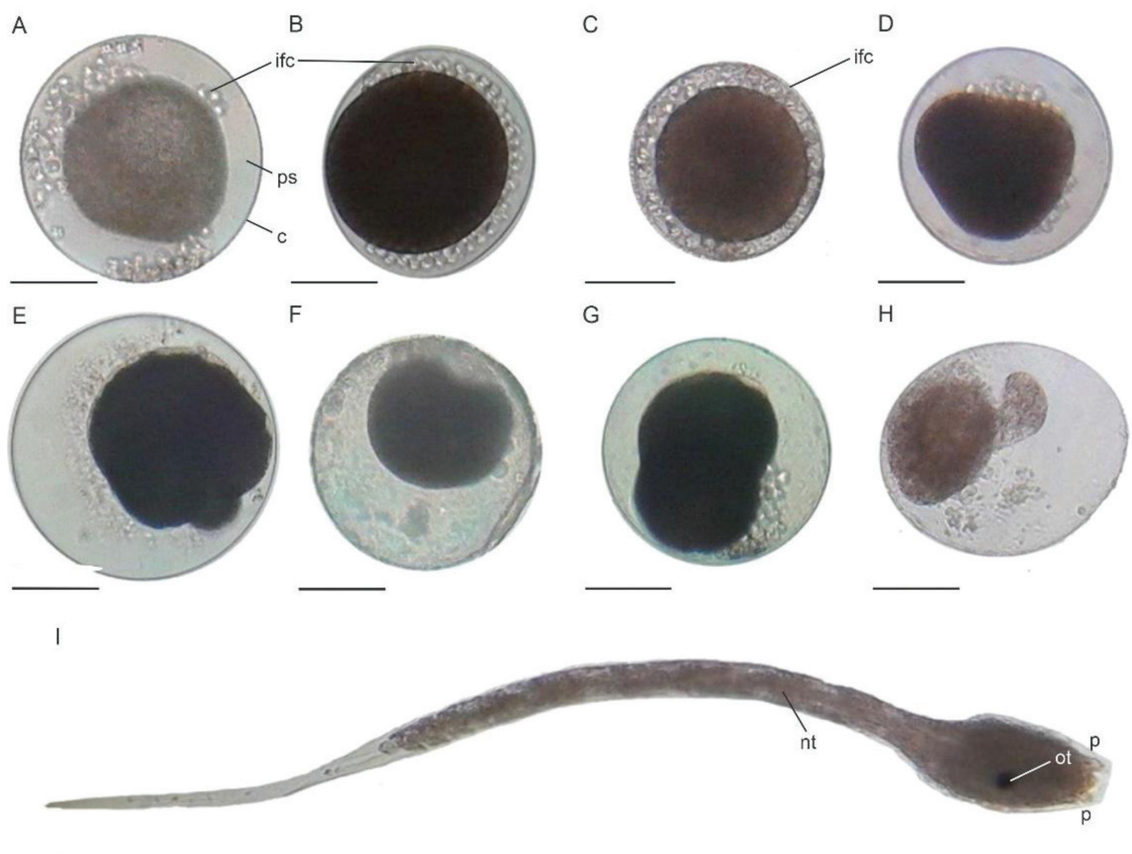

Figure 3. External morphology of developing embryos of *Asterocarpa humilis*. Stages of Hotta *et al*. (2007) are indicated. A. Fertilized egg with a large perivitelline space. B. Stage 1, with the ovum laterally positioned. C. Stage 2, marking the beginning of the cleave period. D. Stage 5. E. Stage 8, the embryo has a square-like shape. F. Stage 10, initial gastrula. G. Stage 14, early neurula with its specific shape. H. Stage 20, early tail bud with the initiation of tail bend. I. Hatching larva. Abbreviations: c= chorion; ifc= inner follicular cells; nt= notochord; ot= otolith; p= papillae; ps= perivitelline space. Scale bar= 100 μm.

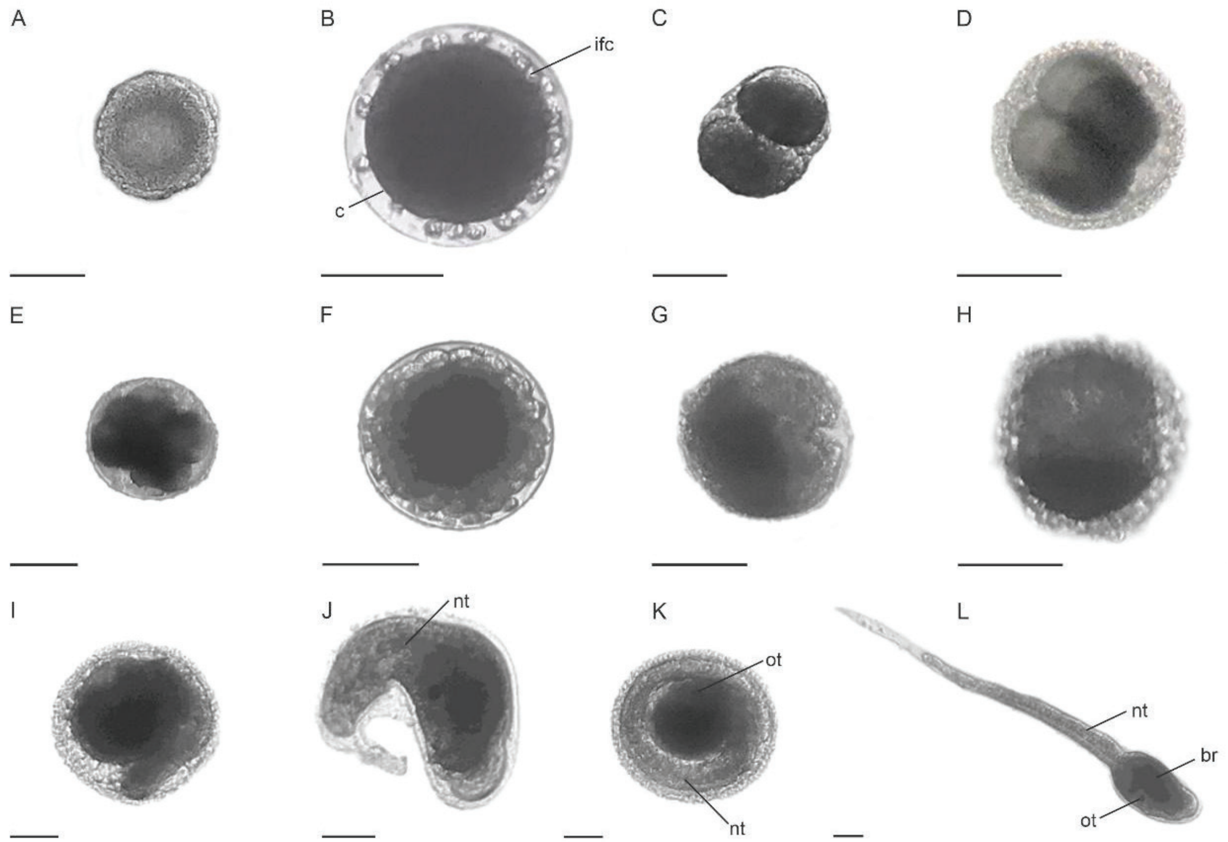

Figure 4. External morphology of developing embryos of *Paramolgula gregaria*. Stages of Hotta *et al*. (2007) are indicated. A. Egg. B. Fertilized egg with increased perivitelline space with inner follicular cells. C. Stage 2, with two first cleavage plane separates the left and right halves of the embryo. D. Stage 3, the second cleavage plane separates the anterior from the posterior halves of the embryos. E. Stage 8, embryo has an almost circle shape. F. Stage 9, indicates the end of the cleavage period. G. Stage 10, gastrula. H. Stage 14, early neurula, the embryo has a characteristic oval shape. I. Stage 20, early tail bud stage in which the tail bends. J. Stage 22, the body adopts a half circle shape. K. Stage 24, the body curves in a circular shape and the tail is most acutely bending; the otolith is discernible. L. Hatching larva. Abbreviations: br= brain; c= chorion; ifc= inner follicular cells; nt= notochord; oc= ocellus; ot= otolith; p= papillae. Scale bar= 100 µm.

chronization with favorable conditions for larval release (Hoegh-Guldberg & Pearse, 1995). Therefore, the differences observed in *P. gregaria* may reflect analogous developmental plasticity associated with local conditions in northern Patagonia. In parallel, *A. humilis* shows delayed perivitelline space formation, distinguishing it from both *C. robusta* and *P. gregaria*.

The development time across the three species varied notably. The embryonic period in *C. robusta* was shorter (~5 h) compared to *P. gregaria* (~18 h), a difference that remained consistent regardless of temperature adjustments. This aligns with findings for *C. intestinalis*, where developmental rates are known to accelerate at higher temperatures but exhibit robust temporal staging at standardized conditions (Hotta *et al*., 2007).

The prolonged development observed in *P. gregaria*, particularly during the neurula and tailbud stages, may be related to the species' adaptation to cooler environments, as indicated by its higher survival rate at 9°C. In contrast, *A. humilis* exhibited minimal variation in egg size during early stages, which may limit its plasticity across different environmental conditions. Morphological differences, particularly in tail and trunk ratios during the tailbud period, were significant. *Ciona robusta* showed a more streamlined morphology, with a higher tail-to-trunk length ratio, resembling descriptions of *C. intestinalis* larvae (Hotta *et al*., 2007). This streamlined body plan is associated with enhanced swimming efficiency, facilitating larval dispersal and the finding of appropriate habitats, a critical trait for invasive success in marine environments (Hodin *et al*., 2018; Hoyer *et al*., 2024). Conversely, the shorter tail and larger trunk observed in *P. gregaria* suggest a trade-off favoring attachment and settlement efficiency over dispersal (Huber *et al*., 2000), consistent with its native status and adaptation to stable habitats. While the larval tail is a defining feature of chordates, enabling active dispersal, its absence or reduction in some Stolidobranch species (Molgulidae) suggests an alternative ecological strategy that prioritizes settlement over motility (Berrill, 1931; Huber *et al*., 2000; Fodor *et al*., 2021). This adaptation likely reflects evolutionary pressures in stable, localized habitats, where dispersal via swimming larvae is less advantageous compared to direct attachment to a suitable substrate, in proximity to other individuals of the same population.

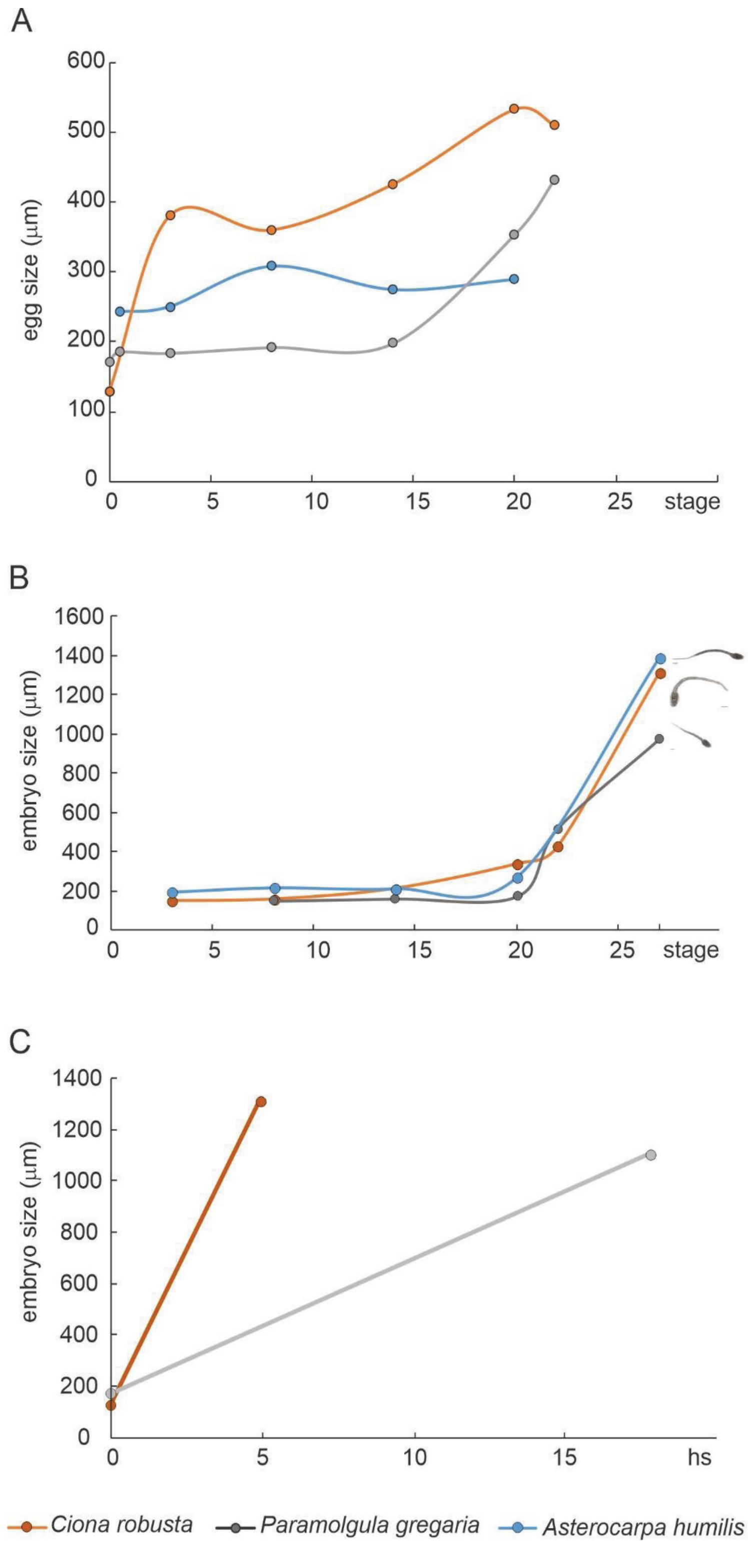

Figure 5. Size variation during the embryonic period of *Ciona robusta* (orange)*, Paramolgula gregaria* (grey), and *Asterocarpa humilis* (blue). A. Variation in egg size across embryonic developmental stages. *Ciona robusta* exhibits a rapid increase in size during early development, followed by a second increment during the cleavage period. *Paramolgula gregaria* shows a more gradual size increase, starting during the neurula stage. In *A. humilis*, the egg size remains relatively stable across development. B. Embryo size at corresponding stages reveals a divergence during the tailbud period, where growth accelerates, ultimately leading to different larval sizes. C. Summarized diagram of embryo size as a function of time (hours post-fertilization) highlighting rapid growth rate in *C. robusta* compared to *P. gregaria*.

The reduced dispersal potential associated with short-tailed larvae may influence the biogeographic patterns and ecological niche of *P. gregaria*. Species with non-motile larvae often exhibit patchy distributions, closely tied to specific substrates or environmental conditions (Zhan *et al*., 2015; Dias *et al*., 2021). This contrasts with motile larvae like those of *C. robusta*, which facilitate colonization across diverse and broad spatial ranges, including artificial habitats such as docks and aquaculture structures (Hirose & Sensui, 2021; Giachetti *et al*., 2022; Hoyer *et al*., 2024).

The embryological transparency of *A. humilis* and its pronounced perivitelline space formation are features shared with *Ascidiella aspersa* (Müller, 1776), another widely distributed species (Shito *et al*., 2020; Funakoshi *et al*., 2021). These traits have been postulated as a form that may enhance its adaptability by improving oxygen diffusion and resistance to hypoxic conditions, thus contributing to its potential for colonization across diverse habitats (Berrill, 1930; Marshall & Bolton, 2007). Its trunk morphology during the tailbud period was distinct, with a more pronounced anterior trunk (Hoyer *et al*., 2024). This may provide a competitive edge in colonizing available substrates in new environments.

The ability of *A. humilis* to thrive in diverse environments, coupled with its reproductive and developmental traits, suggests a high potential for ecological success and possibly invasive behavior (Bishop *et al*., 2012). Its larval morphology, settlement mechanisms, and physiological resilience could allow it to exploit niches in areas disturbed by human activity, as seen in other species (Marins *et al*., 2010; Bishop *et al*., 2012; Nydam *et al*., 2022). Understanding these traits is essential for predicting its ecological impacts and managing its spread in regions, such as the Patagonian coasts, where it is considered cryptogenic and/or its invasive potential remains unclear (Taverna *et al*., 2021).

The observed interspecific differences in embryonic development have direct implications for the species' ability to disperse, colonize, and invade new habitats. Rapid development and motility in *C. robusta* larvae can enhance their highly invasive potential (Robinson *et al*., 2017). In a close area (Puerto Madryn), the species showed mature gonads throughout the year, similar to its native range (Giachetti *et al*., 2022). These traits, combined with its tolerance to a wide range of environmental stressors, make *C. robusta* an effective colonizer of artificial structures, as seen in other *Ciona* species (Robinson *et al*., 2017; Chen *et al*., 2018). The slower development and reduced motility of *P. gregaria* larvae suggest an evolutionary trade-off favoring localized settlement over extensive dispersal. In the case of *A. humilis*, its intermediate traits and adaptability to varied conditions underscore its ability to colonize different habitats and its invasive potential. The species is considered exotic in the North Atlantic after its appearance just over two decades ago, being cryptogenic in areas characterized by temperate-cold waters throughout the southern hemisphere (Taverna *et al*., 2021).

Incubation is a mechanism that also potentially favors the success of the larvae in *A. humilis*. Brooding species retain embryos and can self-fertilize. After hatching, larvae remain in the atrium until they are able to exit through the atrial siphon, being able to disperse for a short time and finally settle (Lambert *et al*., 1995). Ovoviviparous reproduction in solitary ascidians is rare. Incubation decreases the dispersal capacity of gametes, embryos or even larvae (Lambert, 2005). This is an essential factor that also influences the degree of genetic differentiation between populations (Bradbury *et al*., 2008). However, it can present advantages in the case of reduced populations after the transport of propagules by human means through disjunct areas (Alurralde *et al*., 2013; Castilla & Guiñez, 2000). Likewise, incubation reduces the time that gametes and embryos remain in the water, avoiding their potential risk of predation.

Understanding embryonic development is crucial for predicting the ecological success and invasive potential of ascidians. The staging tables developed in this study, combined with descriptive morphological and temporal analyses, provide a framework for assessing how developmental traits contribute to species distribution and adaptability. Such data are invaluable for future studies on invasion biology, as they enable the identification of key traits associated with invasive success, such as rapid development, dispersal efficiency, and environmental tolerance.

CONCLUSIONS

Besides the description of the embryonic development of *C. robusta*, the study contains unprecedented information on *P. gregaria* and *A. humilis,* revealing interspecific variations in fertilization and reproductive success, development time, growth, and morphological traits. In the case of *C. robusta*, rapid development to larva implies less free time and risk of predation. Although the development of *P. gregaria* is slower, a higher success of embryos to the mobile larval stage is an advantageous characteristic in relation to the other species studied. Unlike other Molgulidae, the larva of *P. gregaria* is a short-tailed urodele, which suggests mobility and displacement and therefore, a relative potential for dispersal and finding a suitable habitat for the adult. Likewise, the larvae smaller size compared to the other studied species, would result in less energy expenditure and decrease the possibility of being preyed upon. Incubation and delayed release of larvae in *A. humilis* reduce their risk of being preyed upon, despite their large size. The examination of the development of native and invasive ascidians allows us to better understand the mechanisms driving their establishment and spread, particularly the invasive ones, providing essential knowledge for managing their impacts on marine ecosystems.

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