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COMPARATIVE EMBRYOLOGY AND MUSCLE DEVELOPMENT OF POLYCLAD
FLATWORMS (PLATYHELMINTHES: RHABDITOPHORA)

BY

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BS, Universidad Jorge Tadeo Lozano, 2003

DISSERTATION

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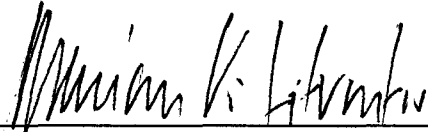
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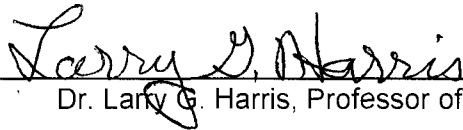
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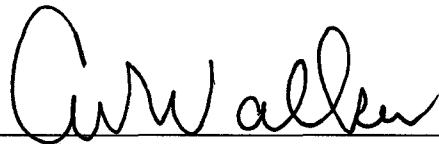
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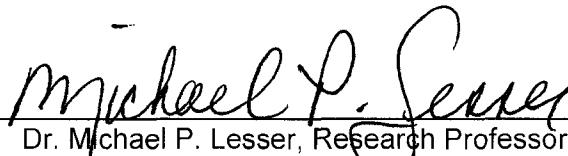
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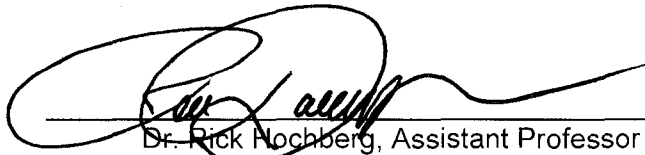
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DEDICATION

To my wonderful parents Ivan and Elena for always being there for me with their unconditional love and support. From thousands of miles away, you have helped me to be where I am today.

To my husband Joey who fills my life with hopes, motivation, and happiness every single day. You have been by my side along the way making all of this worthwhile. You are my everything!

To my unborn little daughter who came recently into my life. She has been my source of motivation and has made the last few months the happiest of my life.

This is because of you and with all my heart I dedicate it to you!

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ABSTRACT

COMPARATIVE EMBRYOLOGY AND MUSCLE DEVELOPMENT OF POLYCLAD FLATWORMS (PLATYHELMINTHES: RHABDITOPHORA)

By

Diana Marcela Bolaños

University of New Hampshire, September, 2008

Polyclads belong to the phylum Platyhelminthes, lineage Rhabdotophora, are simple Bilateria, and represent an interesting and useful group for research in developmental biology. Although polyclads, together with catenulids and macrostomids, have been argued to be most closely related to the ancestral flatworm and hold the key to understand the relationship between development and evolution, knowledge of their embryonic development is still scarce and most of the work on spiralian development has focused on mollusks and annelids. In view of polyclad embryonic significance, a comparative study of their embryonic development including several species of direct and indirect developers was performed.

Developing embryos of 16 species representing 10 families were examined and followed through embryogenesis until hatching. Considerable differences in egg plates, egg capsule morphology, size and number of eggs, and developmental time lines were found among the analyzed species. A correlation between developmental times and morphology of egg capsules was found; likewise it was possible to link larger eggs to direct developing species and longer developmental time and for most but not all, indirect developing species to smaller egg sizes. The number of eggs per egg capsule does not appear to be of systematic value, instead the morphology of the female reproductive system may play a significant role in determining

the number of embryos per egg capsule. The influence of parental care on hatching success was also determined for two local species. Covering of egg masses by the adult was observed for individuals of both species and although this parental care is not necessary for egg development or hatching, it plays a significant role in the hatching success of *Pleioplana atomata* embryos. For individuals of *Imogine zebra*, parental covering of recently laid egg masses may play a role in egg capsule formation.

In the polyclad *Pericelis cata* a case of developmental dimorphism was found. Larvae and juveniles from the same parent hatched simultaneously throughout the three-day hatching period. This represents the first case of true poecilogony reported for polyclad flatworms which may be a bet-hedging strategy in which benthic juveniles are recruited to the parental habitat, and concurrently, siblings disperse as larvae. The most notable feature of the development of *P. cata* was the unusual appearance of extra-embryonic yolk inside the egg capsules. Similarly, almost all the larvae of this species had only one eye, whereas three eyes characterize typical Müller's and Götte's larvae.

Understanding the origin of the muscular system may have implications for the understanding of bilaterian evolution. Hence, a comparative analysis of body wall formation and muscle organization during embryogenesis was performed. Fluorescent dye-conjugated phalloidin was used to characterize the musculature of *Maritigrella crozieri* and *Melloplana ferruginea* which represent an indirect and a direct developing species, respectively. In both species, the first myoblasts were localized in the periphery of the egg. Progressively, myoblasts formed unorganized and rudimentary muscle fibers that further differentiated during development. Muscle differentiation was similar between the two species; however, the process of muscle development progressed quite differently in larvae and juveniles. These results provide additional support that the orthogonal muscle pattern is a symplesiomorphy of Spiralia and it may have been present in the stem species of all Bilateria.

INTRODUCTION

The order Polycladida (formerly considered in the “Turbellaria”) is currently included among the lineage Rhabditophora, within the phylum Platyhelminthes (Ehlers 1986), and represents a highly diverse clade of free-living marine flatworms. Although they represent an ancient lineage, almost nothing is known of their evolutionary history because they have very soft bodies which do not preserve well as fossils. Polyclads are a group of acoelomate, bilaterally symmetrical, and dorsoventrally flattened worms (Hyman 1951), almost exclusively marine with only one species of the genus *Limnostylochus* living in freshwater habitats (Hyman 1951). They can be found living from the littoral to the sublittoral zones (extending to the deep sea) (Quiroga et al. 2006; Quiroga et al. in press), and they commonly dwell on coral and rocky reefs, among shells and seaweeds, as well as on colonial ascidians. Polyclads are of considerable size, ranging from 2-3 mm to several centimeters in length (Hyman 1951). The main characteristic of polyclads is their highly branched intestine from which their name is derived (poly = many; clade = branches) (Fig. 1) (Hyman, 1951).

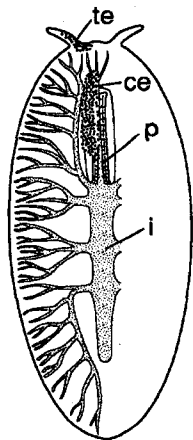


Figure 1. Schematic representation of the highly branched intestine of a polyclad; te: tentacular eyes; ce: cerebral eyes; p: pharynx; i: intestine (from Rieger et al. 1991b).

The order is divided in two suborders: Acotylea and Cotylea. This initial classification is based primarily on the absence or presence of a muscular ventral organ called cotyl or sucker (Lang 1884). Other external traits such as the presence or absence of clusters of eyespots (cerebral, tentacular, marginal) and the presence of either true tentacles or pseudotentacles (folds of the anterior body margin) can be used as systematic characters, too (Newman & Cannon 1994). Morphological characters discernable at the light microscopic level have been most important for the taxonomic classification of the group, particularly the anatomy of the male and female reproductive systems (Faubel 1983, 1984; Prudhoe 1985).

Traditionally, polyclads have been grouped in the Archoophora together with the Catenulida, Acoelomorpha, Macrostomida, and Haplopharyngida (Karling 1967, 1974). This grouping is based on the morphology of the female gonad and the structure of the eggs, features that play an important role in determining the type of embryonic development in platyhelminths. The Archoophora level of organization is considered more primitive and is characterized by homocellular arrangement of female gonads and the production of entolecithal eggs (i.e., yolk is formed from part of the egg itself) (Hyman 1951). This organizational grade contrasts with the Neophora, which are characterized by heterocellular gonads and ectolecithal eggs and include all remaining platyhelminths such as planarians and the parasitic groups.

Polyclads are simultaneous hermaphrodites, with functional male and female reproductive structures. Insemination can occur by true copulation or hypodermic insemination. During true copulation, the sperm is deposited through the female gonopore and probably stored in Lang's vesicle. From there, sperm moves either into the oviducts or the uteri to fertilize the eggs (Hyman 1951; Kato 1940). Hypodermic insemination can be reciprocal or may be unilateral where one animal performs as the

male and the partner animal acts as the female. In this case, one individual injects sperm anywhere through the epidermis of the partner animal, using an armed penis (Hyman, 1951). In dermal impregnation, spermatophores are deposited by the male copulatory organ of one individual onto the dorsal surface of the partner. Deposited sperm are absorbed through the epidermis and then move through the parenchyma to the eggs (Hyman, 1951; Galleni & Gremigni, 1989).

Although polyclads are hermaphrodites, they do not self-fertilize. Regardless of the insemination mode, fertilization takes place in the uterus or in the vagina interna immediately before oviposition (Kato 1940; Prudhoe 1985). Masses of eggs are laid as plates of different shapes and sizes and covered with a sticky gelatinous substance. The number of eggs within each capsule varies depending on the species observed.

Cleavage is holoblastic and spiral; typical of protostomes forming blastomeres quartets, being homoquadratic or slightly heteroquadratic, and resulting in a stereoblastula (Hyman 1951). Gastrulation occurs primarily by epiboly after which the embryo flattens and develops the epidermis and ciliation. Further development of polyclads is either direct or indirect (Fig 2).

Direct development in which eggs hatch directly into juvenile worms is known only among acotyleans. In this type of development, miniature adult hatch with a dorsoventrally flattened body, and a ciliated epidermis with sensory hairs symmetrically arranged along the margin of the body (Lang 1884; Kato 1940; Ballarin & Galleni 1984). Generally, the juvenile worms have two or three pairs of eyes and their internal organs are not developed. The mouth is closed during the entire embryonic development and for a few days post-hatching (Kato 1940). Indirect development involves a free-swimming larva, and has been observed in cotyleans and some acotyleans. During indirect development, eggs can hatch into either of two kinds of larvae, namely a Götte's

or a Müller's larva (Fig. 2) (Hyman 1951; Prudhoe 1985). Müller's and Götte's larvae are ciliated and bear eight or four lobes, respectively. The lobes are distributed around the body and are heavily ciliated. The cilia are used in locomotion and in the production of a feeding current. The mouth lies between the two ventrolateral lobes and behind the oral lobe which is also called an oral hood. Three eyes and the presence of apical and posterior tufts characterized the polyclad larvae (Kato 1940; Hyman 1951; Ruppert 1978).

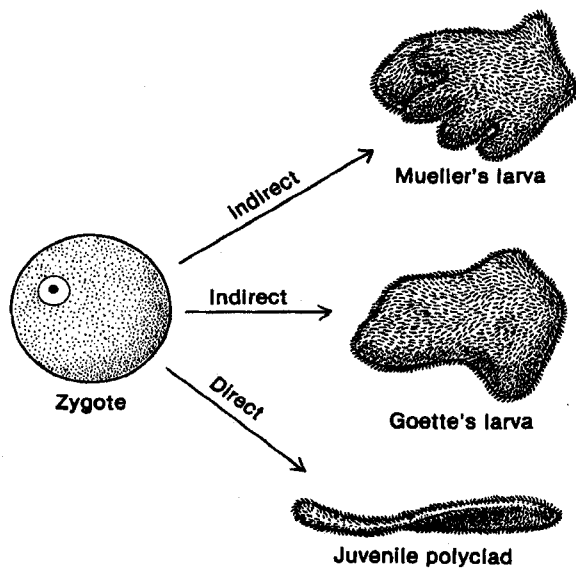


Figure 2. Polyclad development. Schematic representation of indirect and direct development (from Ruppert & Barnes 1994).

Müller's larvae are planktotrophic and has been compared to the trochophore of higher Spiralia (Jägersten 1972; Ruppert 1978). Götte's larvae are considered to be primarily lecithotrophic, although planktotrophic forms can also occur (Ruppert 1978). To date, this type of larva has been described for only a few acotyleans (Kato 1940; Anderson 1977; Murina et al. 1995). It was considered a transitory form of a Müller's larva by Lang (1884). However, Kato (1940) affirmed that the Götte's larva is an independent larval form which metamorphoses into a juvenile worm without ever increasing the number of the lobes. Metamorphosis is completed when miniature worms, only a few millimeters in length, enter the benthic phase of life (Hyman 1951).

Kato (1940) also reported a unique developmental mode in the Japanese species *Planocera reticulata*. In this species, miniature adults hatch after completing metamorphosis via a Müller's larva inside the egg capsule. This phenomenon has been called "intermediate or intra-capsular development" and it is the only variation in development known for polyclads so far.

The Polycladida represents a key group for the understanding of animal development in general, and the life cycle of the stem species of the Bilateria. Not only do they represent an early lineage within the Lophotrochozoa, but they exhibit ancestral character traits in their development (e. g., entolecithal eggs, quartet spiral cleavage) (Thomas 1986; Boyer 1987) in addition to showing three different patterns of development (direct, indirect, intracapsular). Thus, an understanding of their embryonic development is crucial for inferring evolutionary milestones, such as cephalization and bilateral symmetry.

During their benthic life, two major modes of locomotion are observed in polyclads, namely swimming and creeping. Swimming is accomplished by the alternate expansion and contraction of the body margin, similarly to the rippling of lateral fins. During creeping, undulating waves of contractions ripple along the ventral side of the animal, while at the same time, the anterior end tends to be slightly raised off the substrate (Prudhoe 1985). Polyclads muscular system follows the typical musculature of the rhabditophoran model (Tyler & Hooge 2003), although some modifications exist. In general, the rhabditophoran musculature is comprised of a subepidermal (body wall) and a parenchymal component (Hyman 1951). The body wall musculature is located just beneath the epidermis and consists of an outer layer of circular muscles and an inner layer of longitudinal muscles with one or several sheets of diagonally oriented muscle fibers located between the circular and longitudinal layers (Fig. 3). Parenchymal musculature is formed by muscle fibers traversing the central parenchyma and consists

of dorsoventral, transverse, and longitudinal muscles (Fig. 3) (Hyman 1951). Of these, it is the dorsoventral muscles that are often the most prominent.

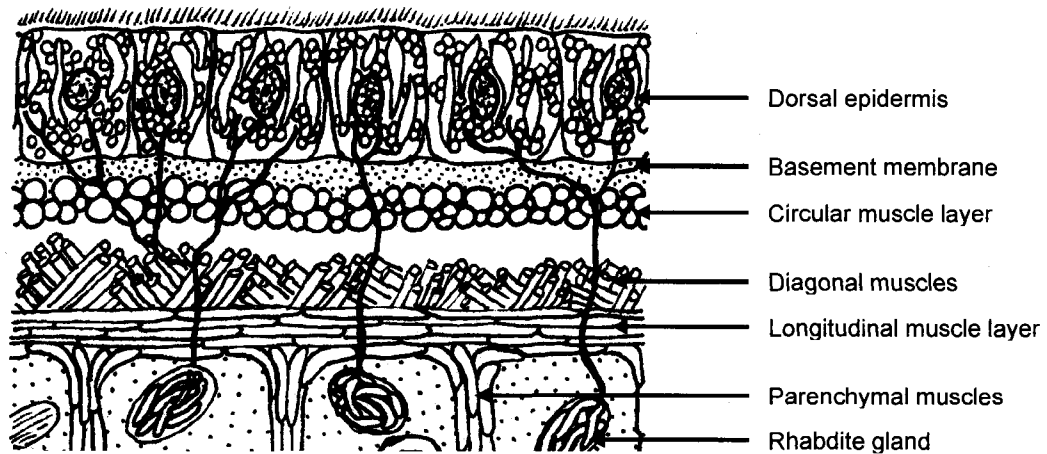


Figure 3. Schematic cross section through the dorsal body wall of a rhabditophoran model (modified from BIODIDAC Image Bank, <http://biodidac.bio.uottawa.ca/>).

The musculature also plays an important role in the development and evolution of the Platyhelminthes. It is assumed that the ancestor of the Bilateria exhibited a pattern of an orthogonal grid of circular and longitudinal muscles; however, the origin and arrangement of this complicated adult body wall musculature is still unclear. Patterns of body wall musculature have been demonstrated to be useful characters for taxonomy (Tyler & Hyra 1998; Hooge & Tyler 1999a) and phylogeny of Acoelomorpha (Hooge & Tyler 1999b; Hooge 2001; Gschwentner et al. 2003), Catenulida (Hooge 2001), and a few select groups within the Rhabditophora (Rieger et al. 1991a, Hooge & Tyler 1999b, Tyler & Rieger 1999, Hooge 2001). Even though descriptions of muscle organization in flatworms have received considerable attention, studies of pattern formation during developmental myogenesis remain scarce (Younossi-Hartenstein & Hartenstein 2000; Hartenstein & Jones 2003; Reiter et al. 1996).

This dissertation has been divided into four chapters beginning with an extensive comparison of the embryonic development of 16 different species of polyclads, based on

morphological traits and their significance for polyclad relationships. The second chapter presents variations in the type of reproduction, egg production, development and the effects of parental care on hatching success for the two local polyclad species, *Pleioplana atomata* and *Imogine zebra*. Chapter 3 reports the first evidence for poecilogony for the cotylean *Pericelis cata*. The last chapter compares the embryonic development and organization of the muscular system of a direct and indirect developing species. Finally, this project involved extensive taxonomic work to identify the species included in this study. During this study, new polyclad species were found and have been described. The description of six new species has been included as appendices which have been published already (Bolaños et al. 2006, 2007). Because the chapters of this dissertation have been prepared as stand-alone manuscripts, there may be some overlap with respect to methodology among the various chapters.

CHAPTER I

COMPARATIVE EMBRYOLOGY OF POLYCLADIDA

Introduction

Polyclads are simultaneous hermaphrodites with separate and well developed male and female systems. Members of this group reproduce sexually either via hypodermic insemination or true copulation; self-fertilization does not occur (Hyman 1951). Embryonic development is generally direct or indirect via a larva. A third type referred to as intermediate or intra-capsular development has been reported for one species (Kato 1940). Direct development is found exclusively among the Acotylea (polyclads without a ventral sucker; Lang 1884), whereas indirect development is the rule for Cotylea, and also has been described for a few acotyleans (Ballarin & Galleni 1984).

Two major larval types have been described for polyclads, namely four-lobed Götte's or eight-lobed Müller's larvae, both of which resemble the trochophore larva (Lang 1884; Kato 1940; Hyman 1951). Both types are completely ciliated, have an apical tuft of cilia and a band of longer, denser cilia that encircles the body along the sides of the lobes. Larvae are planktonic for a few days to weeks and upon settling, metamorphosis is completed, with a juvenile adult of only a few millimeters in length entering the benthos (Hyman 1951).

Studies of polyclad development date to early investigations of polar body formation, fertilization and cleavage patterns (Hallez 1879; Selenka 1881; Götte 1878, 1882). Lang (1884) and Surface (1907) provided detailed descriptions of spiral cleavage and germ layer formation in *Discocelis tigrina*, and *Hoploplana inquilina*, respectively. Since then, different aspects of embryogenesis and hatching have been recorded for a number of polyclads (Kato 1940; Christensen 1971; Lytwyn & McDermott 1976; Anderson 1977; Ballarin & Galleni 1984). Additionally, polyclads have been used as models to understand spiralian development (Boyer & Henry 1995; Henry et al. 1995). More recently, *Hoploplana inquilina* and *Imogine mcgrathi* have become the organisms of choice for cell lineage tracing using fluorescent and immunohistochemical approaches (Boyer et al. 1996, 1998; Younossi-Hartenstein & Hartenstein, 2000).

The present chapter constitutes a comparative analysis of the development of six species of acotylean and ten species of cotylean flatworms, representing direct and indirect developing species. The major goal of this study is to compare characteristics of polyclad embryogenesis that as of yet had not been described and to determine their significance for polyclad systematics.

Materials and Methods

Animal Collection

Polyclad specimens representing 10 families were hand-collected from under rocks in the intertidal and shallow subtidal zones of the Atlantic coast of Florida and New Hampshire, and the Caribbean coast of Colombia. Species examined and collection localities with georeferences are listed in Table 1. Specimens of *Imogine zebra* were bought from the Marine Biological Laboratory (Wood's Hole, Massachusetts, USA), where they had been collected from either whelks or moon shells that were inhabited by hermit crabs.

Table 1. Taxonomic list of polyclad species examined during the study and their respective collection localities. *MBL*, Marine Biological Laboratory, Woods Hole, Massachusetts; *SIFL*, Sebastian Inlet, Florida (27° 51' 25.149 N; 80° 26' 45.0342 W); *PIFL*, Peanut Island, Florida (26° 46.428' N; 80° 82.608' W); *OPNH*, Odiorne Point, New Hampshire (43° 02.395' N; 70° 42.899' W); *SMCOL*, Inca-Inca, Gaira Bay Santa Marta, Colombia (11° 11' N; 74° 14' W).

Taxon	Species	Collection Locality
Suborder: Acotylea		
Family: Stylochidae	<i>Imogine zebra</i> (VERRILL 1882)	MBL
Family: Notoplanidae	<i>Notocomplana lapunda</i> (MARCUS & MARCUS 1968)	SIFL
Family: Pleioplanidae	<i>Pleioplana atomata</i> (MÜLLER 1776)	OPNH
	<i>Melloplana ferruginea</i> (SCHMARDA 1859)	PIFL; SMCOL
Family: Gnesioceridae	<i>Styloplanocera fasciata</i> (SCHMARDA 1859)	PIFL; SMCOL
Family: Stylochoplanidae	<i>Armatoplana lactoalba</i> (VERRILL 1900)	SIFL
Suborder: Cotylea		
Family: Pseudocerotidae	<i>Pseudoceros bicolor</i> VERRILL 1902	SIFL
	<i>Phrikoceros mopsus</i> (MARCUS 1952)	SIFL; SMCOL
	<i>Thysanozoon brocchii</i> (RISSO 1818)	SIFL
Family: Pericelidae	<i>Pericelis cata</i> MARCUS & MARCUS 1968	SIFL; SMCOL
Family: Boniniidae	<i>Boninia divae</i> MARCUS & MARCUS 1968	SMCOL
Family: Euryleptidae	<i>Cycloporus gabriellae</i>	SIFL; SMCOL
	<i>Maritigrella crozieri</i> (HYMAN 1939)	SIFL; SMCOL
Family: Prosthlostomidae	<i>Enchirdium periommatum</i> BOCK 1913	SIFL; SMCOL
	<i>Prosthlostomum lobatum</i> PEARSE 1938	SIFL; SMCOL
	<i>Prosthlostomum pulchrum</i> BOCK 1913	SIFL; SMCOL

Using a soft paintbrush to avoid autolysis, animals were lifted off the substrate, and were placed individually into small plastic bags filled with seawater.

Culture

Reproductive maturity of individuals was determined by inspecting their ventral sides for eggs, which are visible in the oviducts. Oviparous specimens were placed into individual plastic bags, containing Millipore-filtered seawater, and the water was changed every other day. Individuals laid batches of eggs along the sides of the plastic bags. Egg masses were measured, dated, and labeled externally with a permanent marker. The portions of the plastic bags containing the masses were cut out and placed into individual 500 ml glass containers, assuring that the sides of the plastic pieces with the attached eggs were in constant contact with seawater. To follow development, the plastic pieces were transferred to glass slides three times daily, covered with a few drops of seawater, and the eggs were observed and measured under a Leica DMLB microscope equipped with a Nikon CoolPix 8700. All changes were photographically documented. Developing embryos were followed through embryogenesis until hatching. Feeding trials were performed with larvae of *Cycloporus gabriellae*. The larvae were reared in 1000 ml glass containers that were kept at room temperature. Immediately after hatching, they were fed with the microalga *Isochrysis* sp.

Because of a potential link between temperature and developmental time lines, culture temperatures are given in Table 4. Additionally, to test for a correlation between developmental time and egg size a multivariate analysis was performed using the statistical software JMP®7.

Scanning Electron Microscopy (SEM)

For scanning electron microscopic (SEM) observations, developing eggs, larvae, and juveniles were fixed in 2.5% glutaraldehyde for 2 h, post-fixed in 1% OsO₄ in

cacodylate buffer for 1 h, and dehydrated in an ethanol series. Specimens were critical point dried, mounted on SEM stubs, sputter coated with gold and observed with a JEOL JSM-6400V SEM (Smithsonian Marine Station, Fort Pierce, Florida).

Histology

For taxonomic identifications, adult specimens were fixed in Petri dishes containing frozen 10% buffered formalin after a modified protocol of Newman & Cannon (1995). Animals were covered completely with additional fixative and smoothed with a paintbrush to assure their flatness. Following fixation, segments measuring about 6 mm x 4 mm and containing the reproductive structures were dissected. The segments were dehydrated through an alcohol series, embedded in paraffin, and sagittally sectioned (5-7 μ m) using an AO Spencer E800 microtome. Finally, sections were stained with hematoxylin and eosin, and mounted in Permount on glass slides. The remaining parts of the worms were dehydrated, cleared with HistoClear and mounted in Permount as whole mounts. Reconstructions of the reproductive systems were derived from sectioned material and whole mounts, and taxonomic identifications were based on Faubel (1983-1984).

Results

Egg Plates (Figs. 4A-E)

Some worms started laying egg plates immediately, whereas other species laid egg masses only several days post-collection. Most species deposited 3-5 plates, one of which was used as a standard and the remaining egg plates were considered replicates. An individual egg plate consisted of eggs enclosed in a hard egg capsule of varying morphologies several of which were surrounded completely by a gelatinous substance. The size and arrangement of egg masses differed between species (Table 2; Fig. 4A-E).

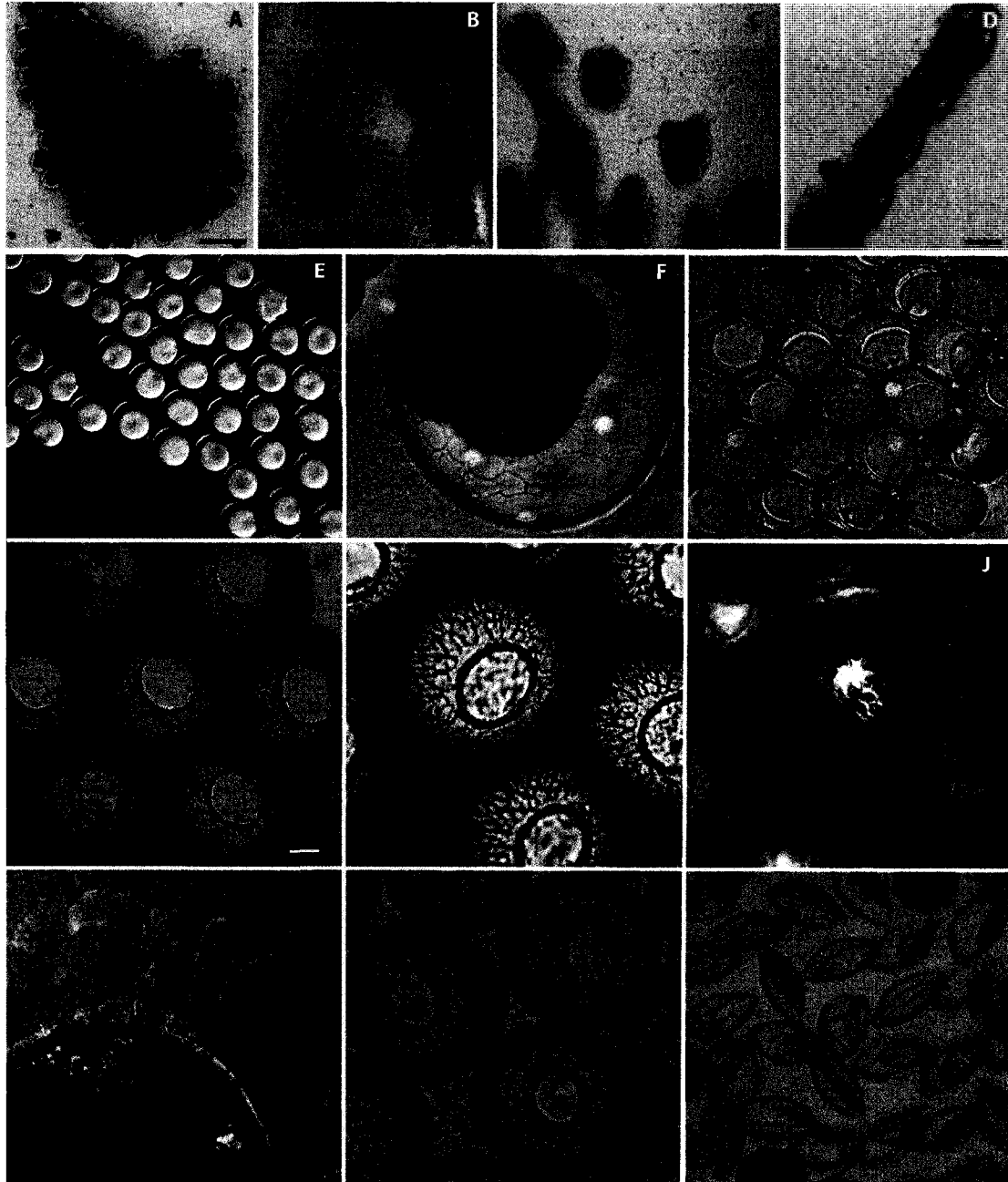


Figure 4. Egg plate and capsule arrangements in various polyclads. (A) Typical egg plate, irregularly disc shaped with tightly packed egg capsules. Scale bar, 500 μ m; (B) egg plates with evenly spaced capsules. Scale bar, 1 mm; (C) small batches of eggs forming a single mass in *Melloplana ferruginea*. Scale bar, 500 μ m; (D) single row of eggs in *Boninia divae* with more than one egg per capsule; (E) single encapsulated egg of *Styloplanocera fasciata*. Scale bars, 150 μ m; (F) detail of egg capsule surface in acotyleans sculptured with small rings; (G) smooth-surfaced egg capsule with a full-size operculum of *Phrikoceros mopsus*; (H) smooth-surfaced opaque egg capsules with a small operculum of *Enchiridium periommatum*; (I) egg capsules of *Prosthiosomum lobatum* with fissures forming asymmetrical shapes; (J) opercular detail of egg capsule of *P. lobatum* with small scattered spots; (K) gelatinous cover surrounding the eggs with scale pattern in *Prosthiosomum pulchrum*; (L) egg capsule of *P. pulchrum* with a fractured appearance; (M) empty flattened egg capsules of *Cycloporus gabriellae*. Scale bars, 50 μ m.

Table 2. Comparison of egg plate size of six acotylean and ten cotylean species.

ACOTYLEA		COTYLEA	
Species	Size (mm)	Species	Size (mm)
<i>Notocomplana lapunda</i>	3 x 2.5	<i>Cycloporus gabriellae</i>	5 x 2
<i>Armatoplana lactoalba</i>	3 x 2	<i>Phrikoceros mopsus</i>	7 x 6
<i>Melloplana ferruginea</i>	10 x 5	<i>Maritigrella crozieri</i>	5 X 3
<i>Styloplanocera fasciata</i>	8 x 5	<i>Pseudoceros bicolor</i>	4 x 3.5
<i>Pleioplana atomata</i>	5 x 3.5	<i>Thysanozoon brocchii</i>	16 x 7
<i>Imogine zebra</i>	2 x 1.5	<i>Pericelis cata</i>	13 x 5
		<i>Prosthlostomum lobatum</i>	3 x 3
		<i>Prosthlostomum pulchrum</i>	5 x 3
		<i>Enchirdium periommatum</i>	6 x 3
		<i>Boninia divae</i>	5 x 3

Generally, egg plates were irregularly shaped discs containing numerous, tightly packed egg capsules. However, in some cases, uniformly spaced egg capsules dispersed throughout the gelatinous matrix were observed as well. For example, in *Melloplana ferruginea* small batches of eggs were combined to form one large plate (Fig. 4C), whereas in *Boninia divae*, an individual row of eggs, sometimes two rows, formed the entire mass (Fig. 4D). In most species, eggs were deposited in a single layer but in *Cycloporus gabriellae* and *Pleioplana atomata*, a second layer of eggs was deposited on top of the first layer. In all but one species of Acotylea, and in members of the Pseudocerotidae, and Euryleptidae, each egg capsule contained a single egg (Fig 4E). The remaining cotylean species examined deposited multiembryonic capsules containing between 2 and 6 eggs per capsule (Table 3; Fig. 4D). With always two embryos per egg capsule, *Enchirdium periommatum* displayed an invariable number. Although most egg capsules of *P. atomata* also contained one embryo only, a few were

housing twins (Table 3); most of these developed into conjoined juveniles that adhered to each other even after hatching (see Chapter II).

Table 3. Comparison of egg size, egg capsule size, and number of eggs per capsule (n=10).

Species	Egg Size (μm) \pm SD	Capsule Size (μm) \pm SD	Number of Eggs/Capsule
Acotylea			
<i>Armatoplana lactoalba</i>	123 \pm 5.6	177 \pm 11.6	1
<i>Notocomplana lapunda</i>	146 \pm 14.2	154 \pm 8.5	1
<i>Melloplana ferruginea</i>	125 \pm 5.4	154 \pm 6	1
<i>Styloplanocera fasciata</i>	121 \pm 3.6	151 \pm 6.7	1
<i>Imogine zebra</i>	203 \pm 14.8	258 \pm 16.8	1
<i>Pleioplana atomata</i>	385 \pm 55.9	484 \pm 42.4	1-2
Cotylea			
<i>Cycloporus gabriellae</i>	116 \pm 6.9	121 \pm 7.9	1
<i>Maritigrella crozieri</i>	134 \pm 3.2	156 \pm 7.2	1
<i>Pericelis cata</i>	103 \pm 5	245 \pm 11.8	3-5
<i>Boninia divae</i>	92 \pm 4.3	230 \pm 10.6	2-6
<i>Pseudoceros bicolor</i>	172 \pm 7.4	172 \pm 7.4	1
<i>Phrikoceros mopsus</i>	87 \pm 5.3	100 \pm 4.2	1
<i>Thysanozoon brocchii</i>	112 \pm 6	119 \pm 6.3	1
<i>Enchiridium periommatum</i>	165 \pm 7.3	229 \pm 10.3	2
<i>Prosthiosomum lobatum</i>	88 \pm 3	230 \pm 14	2-4
<i>Prosthiosomum pulchrum</i>	90 \pm 4.9	214 \pm 16.1	5-6

Initial egg size varied among species and changed as development progressed. The largest variation was observed in species with one egg per capsule; *P. bicolor* deposited the largest eggs, where as *Phrikoceros mopsus* produced the smallest ones. A fewer eggs per capsule with increased egg size could be discerned for *E. periommatum*, however, no such relationship was found for the remaining species

(Table 3). The relationship between egg size and developmental time showed a low correlation coefficient ($\rho = 0.28$) indicating that there is no a significant association between these two variables. .

Egg capsules surfaces (Figs. 4F-M)

Variation was also observed in structural details of egg capsule surfaces. In acotyleans, superficial, spherical shield-like plates were distributed unevenly over the entire surface (Fig. 4F). At hatching, the entire egg capsules fragmented completely and juveniles were released. In contrast, cotyleans produced egg capsules of different textures. However, lid-like opercula on the dorsal side of the egg capsule were a constant feature in all species examined, with the exception of Euryleptidae. During the hatching process, the emerging larvae broke the opercula, but unlike in acotyleans, the remaining parts of the egg capsules stayed intact (Figs. 4G, H). In *P. mopsus* and *T. brocchii*, the egg capsules were characterized by smooth, transparent surfaces, and by opercula with diameters almost equal to that of the egg capsules (Fig. 4G). The egg capsule surfaces of *Pericelis cata*, and *B. divae* consisted of a rough texture of brownish color, and an opercular opening that was about half of the capsule diameter.

Egg capsules surfaces in Prosthlostomidae provided morphological differences among species. Egg capsules of *Enchiridium periommatum* exhibited a smooth opaque surface with a small-sized operculum (Fig. 4H). In *Prosthlostomum lobatum*, the entire egg capsule appeared wrinkled with pronounced edges; over the opercula only, small spots were distributed (Figs. 4I, J). Finally, the eggs capsules of *P. pulchrum* had a fractured surface appearance (Fig. 4K, L), and the gelatinous material of the egg plates appeared scale-like near the margin (Fig. 4K).

Egg capsules of the euryleptids, *Cycloporus gabriellae* and *M. crozieri* were transparent, smooth, and delicate, however, no opercula were present. After hatching, euryleptid egg capsules collapse (Fig. 4M).

Embryogenesis

Cleavage and development of the eggs showed similar progress in all examined species, however, there was a marked variation in the timing of developmental stages (Fig. 5A, B). Cleavage is holoblastic, generally with equal and synchronous divisions during the first two divisions. Thereafter, divisions become unequal, irregular and asynchronous. In some embryos of *Cycloporus gabriellae* cells of the 4-cell stage were unequal early on, with two large and two small blastomeres (Fig. 6A). Likewise, asynchronous development was observed in eggs within the same egg plate (*Melloplana ferruginea*) or within the same egg capsule (*Prosthiostomum lobatum*) that were dividing at different rates (Figs. 6B, C, D). When early unequal divisions produced blastomeres of extremely different sizes, embryos usually did not develop or past the third cleavage division (Fig. 6D, E, F).

To facilitate understanding of embryogenesis, direct-developing and indirect-developing species are described separately. In this study, developmental mode mirrored taxonomic placement into suborders (although indirect developing acotyleans do exist, see Introduction).

Direct Development in Acotylea: Six species of acotyleans representing 5 families were studied (Table 1). These species represent some of the most common polyclads found in rocky intertidal habitats (Quiroga et al. 2004a, b; Rawlinson 2008). Zygotes were approximately 122-150 μm in diameter (Table 3; Figs. 4C, E, 7A). Zygotes reached the 64-cell stage 1-2 days after oviposition, except for *P. atomata* in which the first cleavage occurred on day 3 (Fig. 5A).

The first cleavage plane divided the egg from the animal to the vegetal pole, resulting in two blastomeres of nearly equal size (Figs. 7B, C). Subsequent divisions, forming 4, 8, and 16 cells followed (Figs. 7D-F). Divisions became irregular and difficult to distinguish at the 32- and 64-cell stages (Fig. 7G). At the end of the cleavage

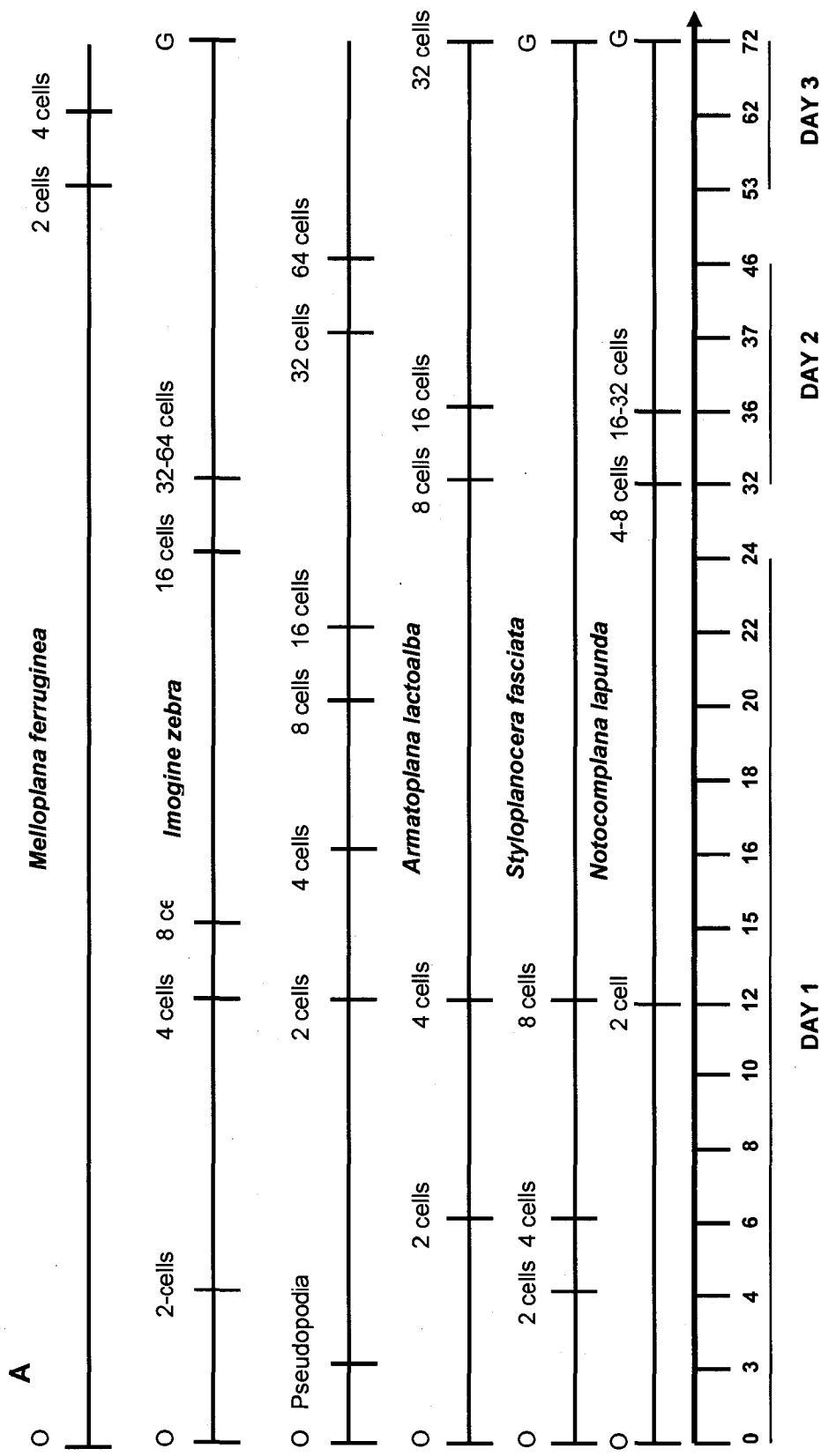
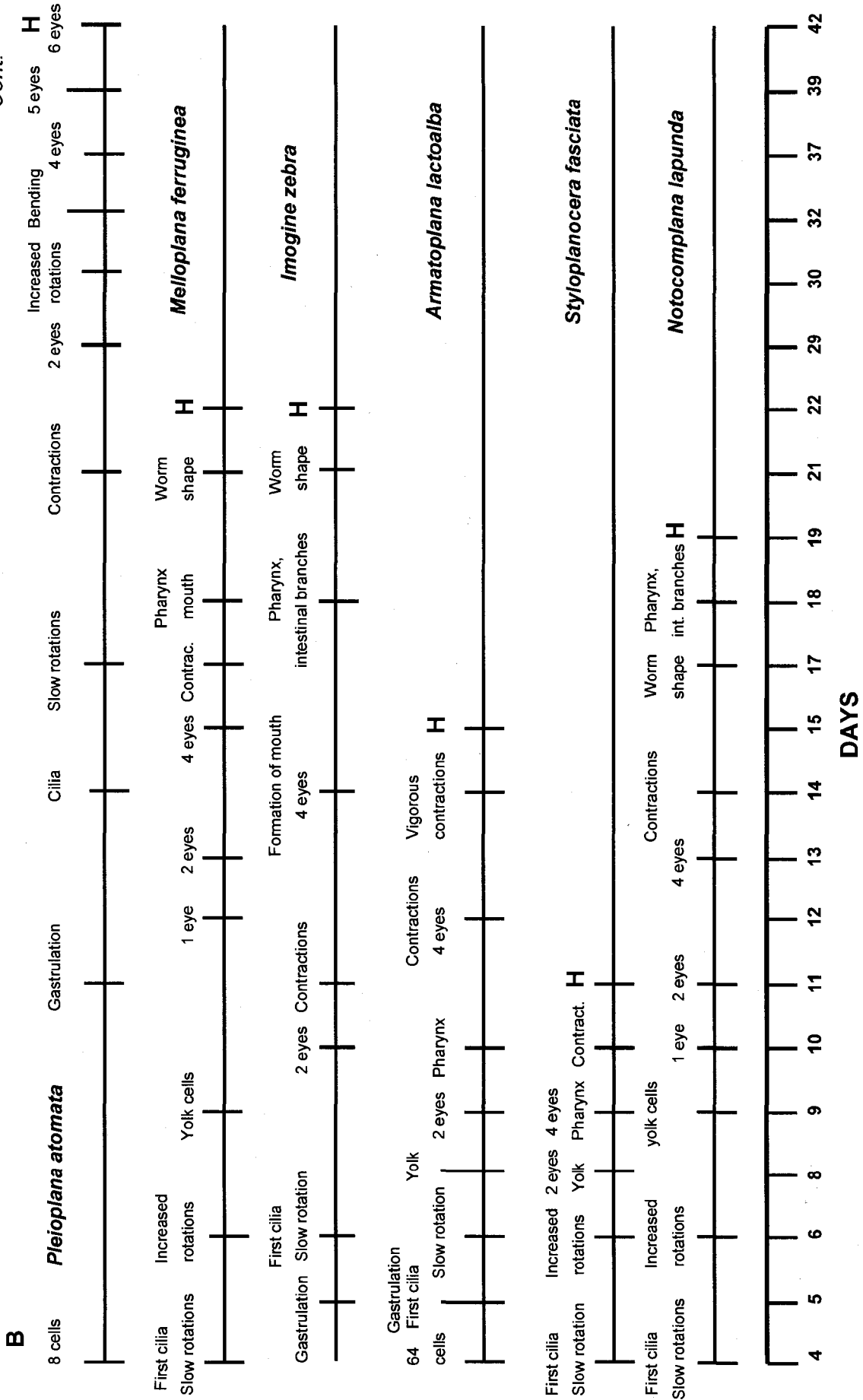


Figure 5. Comparative time line for the embryonic development of six acotyleans. (A) Time line from oviposition to gastrulation, except *A. lactoalba* and *P. atomata*; (B) continuation of development from gastrulation to hatching. O= oviposition, G= gastrulation, H= hatching.

Cont.



the cells of the mesoblast were evident and the ectodermal cells started enclosing the egg (Fig. 7H). Gastrulation occurred by epiboly in all 16 species. Macromeres of the vegetal pole migrated to the interior of the embryo developing into large yolk cells (Figs. 7I, J). Gastrulation resulted in a solid, spherical stereogastrula which soon began slow rotational movements initiated by short cilia that covered the stereogastrula (Fig. 7K). The developing embryos steadily increased in size until they occupied nearly all the space inside the egg capsules. Over the next days, rotational movements increased as cilia grew in length and number. Large yolk droplets were observed to migrate to the interior of the embryo where they were reabsorbed slowly (Fig. 7L).

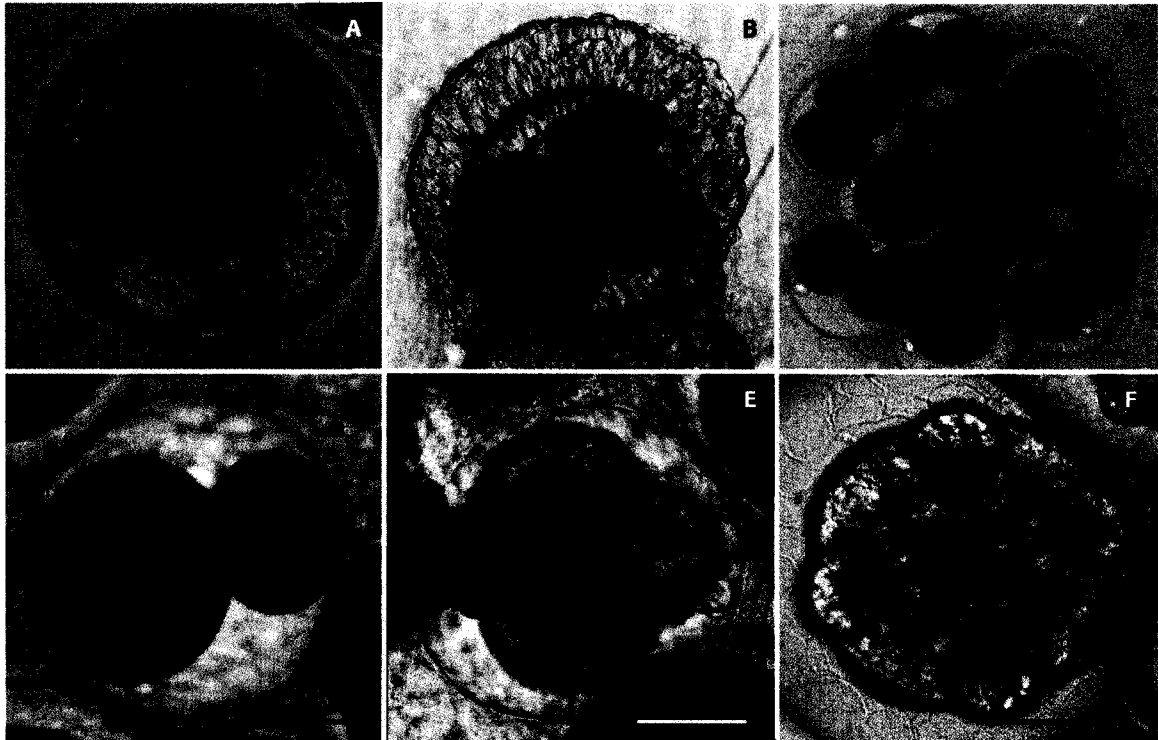


Figure 6. Asynchronous and unequal divisions in polyclad eggs. (A) Four-cell stage in *Cycloporus gabriellae* with two large and two small blastomeres; (B) two zygotes within the same egg capsule in *Prosthlostomum lobatum*, one undivided zygote and the other divided into two blastomeres; (C) single encapsulated eggs in *Melloplana ferruginea*, some eggs still undivided, other with first division completed; (D) first cleavage of a single encapsulated egg, producing two blastomeres of markedly different size; (E) second cleavage of a single encapsulated egg producing two blastomeres of nearly equal size and two of different size; (F) third cleavage of a single encapsulated egg producing one large blastomere. Scale bars, 50 μ m.

Most species formed a single primary eye soon after gastrulation, followed by a second eye a day later (Figs. 7M-O). Subsequently around days 12-15, a second pair of eyes was observed; it is highly likely that the second pair formed by a division of the first pair (Figs. 8A-C). In other species (*Melloplana ferruginea*, *P. atomata*) a third pair of eyes developed and juveniles hatched with a total of 6 eyes (Fig. 8D). In a few specimens of *M. ferruginea* some of the embryos developed a third single eye or a pair of eyes which sank into the mesodermal layer prior to hatching (Fig. 8C, D).

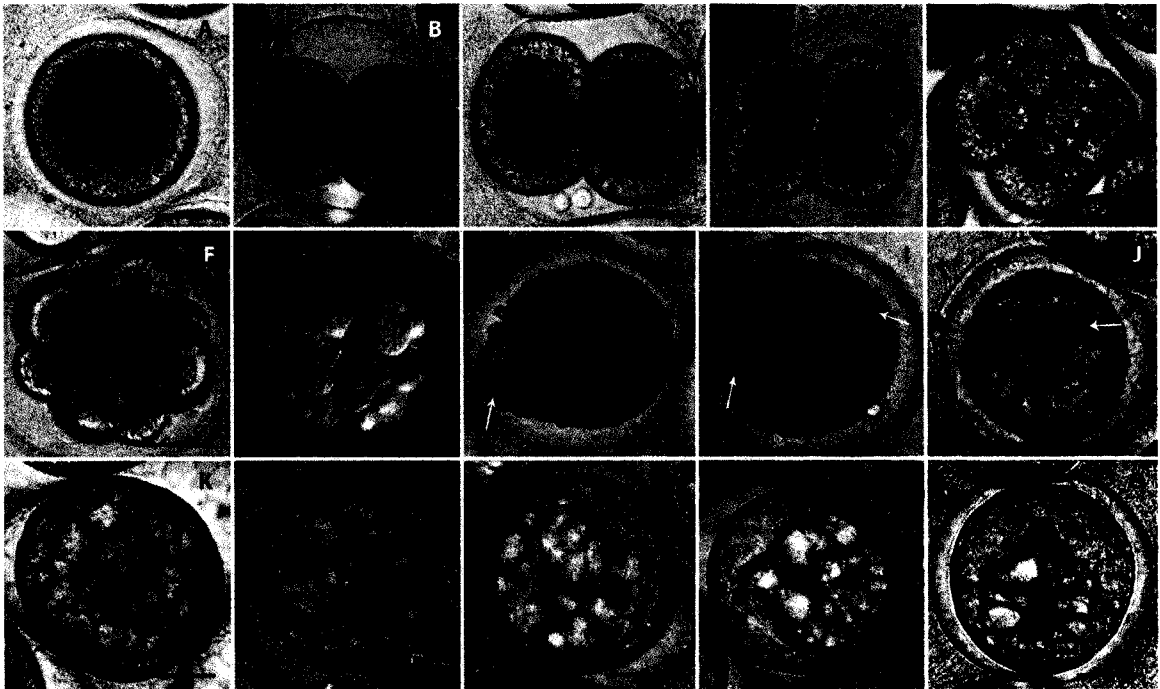


Figure 7. Embryonic development in acotyleans. (A) Zygote; (B) start of first division through animal and vegetal poles; (C) 2-cell stage; (D) start of second division; (E) 4-cell stage. (F) 16-cell stage viewed from the vegetal pole; (G) 64-cell stage; (H) egg before epiboly, arrow indicates mesoblast cell; (I) gastrulation, arrows indicate micromeres migrating towards vegetal pole; (J) arrow indicates formation of blastopore; (K) larger stereogastrula; (L) embryo with large yolk cells; (M) more advanced stage with granular yolk, and presence of a single eye; (N) single eye stage with yolk cells migrating to the center of the embryo; (O) 2-eye stage with yolk and mesodermal cells surrounding the rudimentary pharynx. Scale bars, 50 μ m. (A-F, H, L, M, O) *Melloplana ferruginea*; (G) *Styloplanocera fasciata*; (I, J, K, N) *Notocomplana lapunda*.

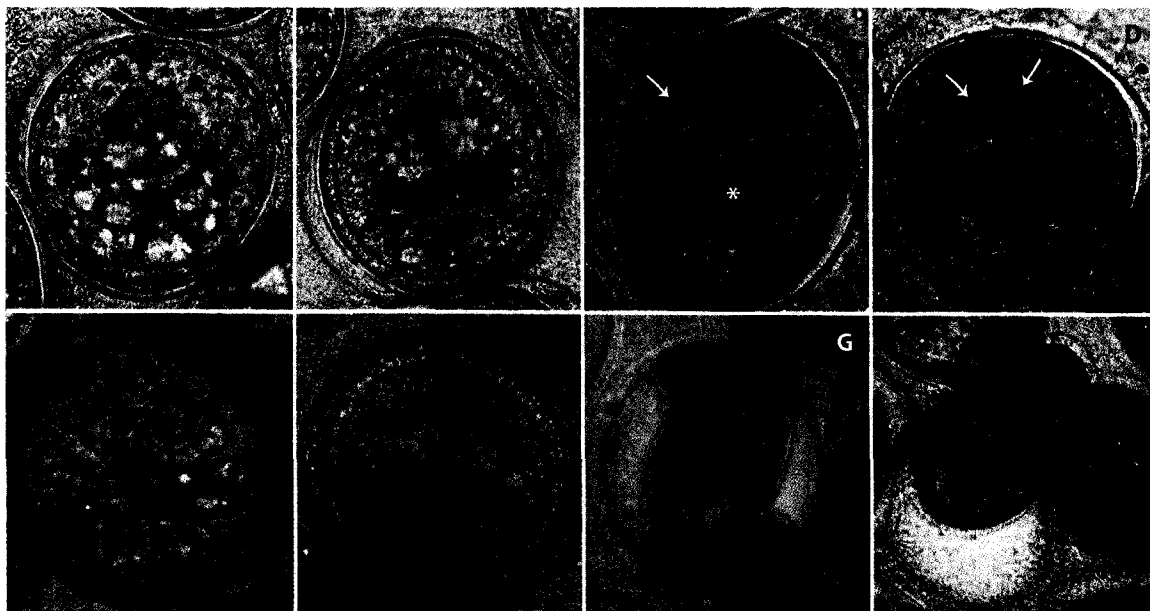


Figure 8. Development of eyes and pharynx in acotylean embryos. (A) 4-eye stage embryo with yolk and mesodermal cells surrounding a rudimentary pharynx; (B) 4-eye stage embryo of increased size and with reabsorbed yolk cells; (C) arrow indicates formation of a fifth eye and dark pigment outlining the pharynx (asterisk); (D) formation of a third pair of eyes, arrows indicating eyes sunk into the mesodermal layer; (E) branches of the intestine and pharynx extending anteriorly to the level of the eyes outlined by yolk cells, dark pigment absent; (F) ventral mouth seen inside the egg capsule; (G) body with elongated shape and contracting before hatching; (H) juvenile before hatching stretching and elongating the egg capsule. Scale bars, 50 μm . (A-D, F) *Melloplana ferruginea*; (G, H) *Notocomplana lapunda*; (E) *Armatoplana lactoalba*

At about day 13, the yolk was absorbed almost entirely, and the mesoderm formed large cell masses posteriorly, surrounding a rudimentary pharynx formed by endodermal cells (Fig. 8A). A pharynx and intestinal branches were evident in some species just prior to hatching. They could be distinguished by dark brown or green pigmentation in the body which in some species (*N. lapunda*, *M. ferruginea*) extended anteriorly to the level of the eyes (Figs. 8B-D). Additionally, in *A. lactoalba* ramifications of the intestine were outlined by yolk cells and brown pigment was absent (Fig. 8E). In *P. atomata* and *M. ferruginea* a large circular mouth was observed at the posterior end prior to hatching (Fig 8F). However, in the majority of species, the mouth was only visible post-hatching. The embryos continued to elongate their bodies and for a few more days they rotated, bent, and contracted inside the egg capsule (Fig. 8G, H). Hatching

occurred 2 or 3 weeks after oviposition, with the exception of *P. atomata*, which hatched in 5 weeks (Fig. 5B; Chapter II).

Indirect Development in Cotylea: Species belonging to the Cotylea are known to develop indirectly via an eight-lobed Müller's larva. The development of ten species representing five families (Table 1) from oviposition to hatching was followed.

In general, indirect developing species tended to hatch in a shorter time period than direct developing species, with the exception of *Pericelis cata* and *Pseudoceros bicolor* in which development was completed in 29 and 20 days, respectively.

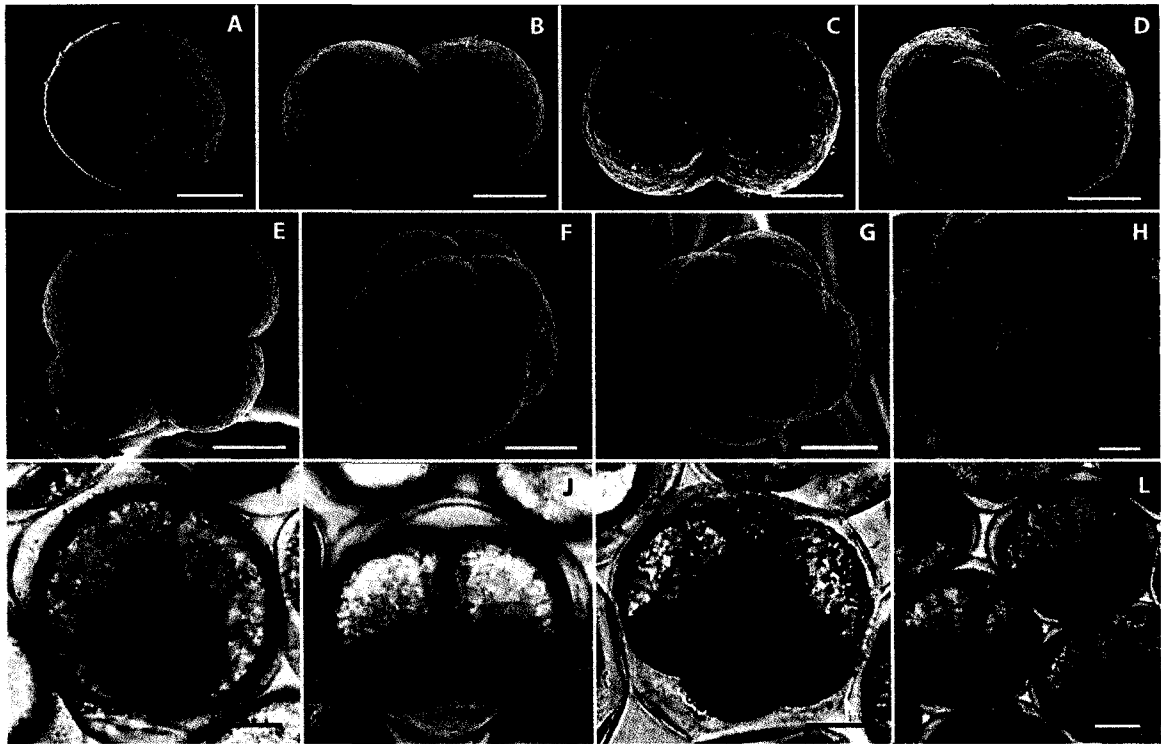


Figure 9. Development in cotyleans. (A-H) Scanning electron microscopy images of the first cleavages and a polar body formation in *Cycloporus gabriellae*. (A) Zygote after oviposition; (B) first division; (C, D) detail of the cleavage furrow through animal and vegetal poles; (E) second division, four-cell stage; (F) side view of third cleavage, producing four macromeres and four micromeres; (G) top view of third cleavage with the formation of a polar body. Scale bars, 25 μm ; (H) detail of the polar body. Scale bar, 5 μm . (I-L) Eggs in *Phrikoceros mopsus* with conspicuous dark coloration typical of pseudocerotids. (I) Zygote after oviposition with conspicuous black area at the lower edge of the egg; (J, K) first and second division with dark area at the periphery; (L) side view of zygotes at third division with four large blastomeres at the vegetal pole and small and dark micromeres at the animal pole. Scale bars, 25 μm .

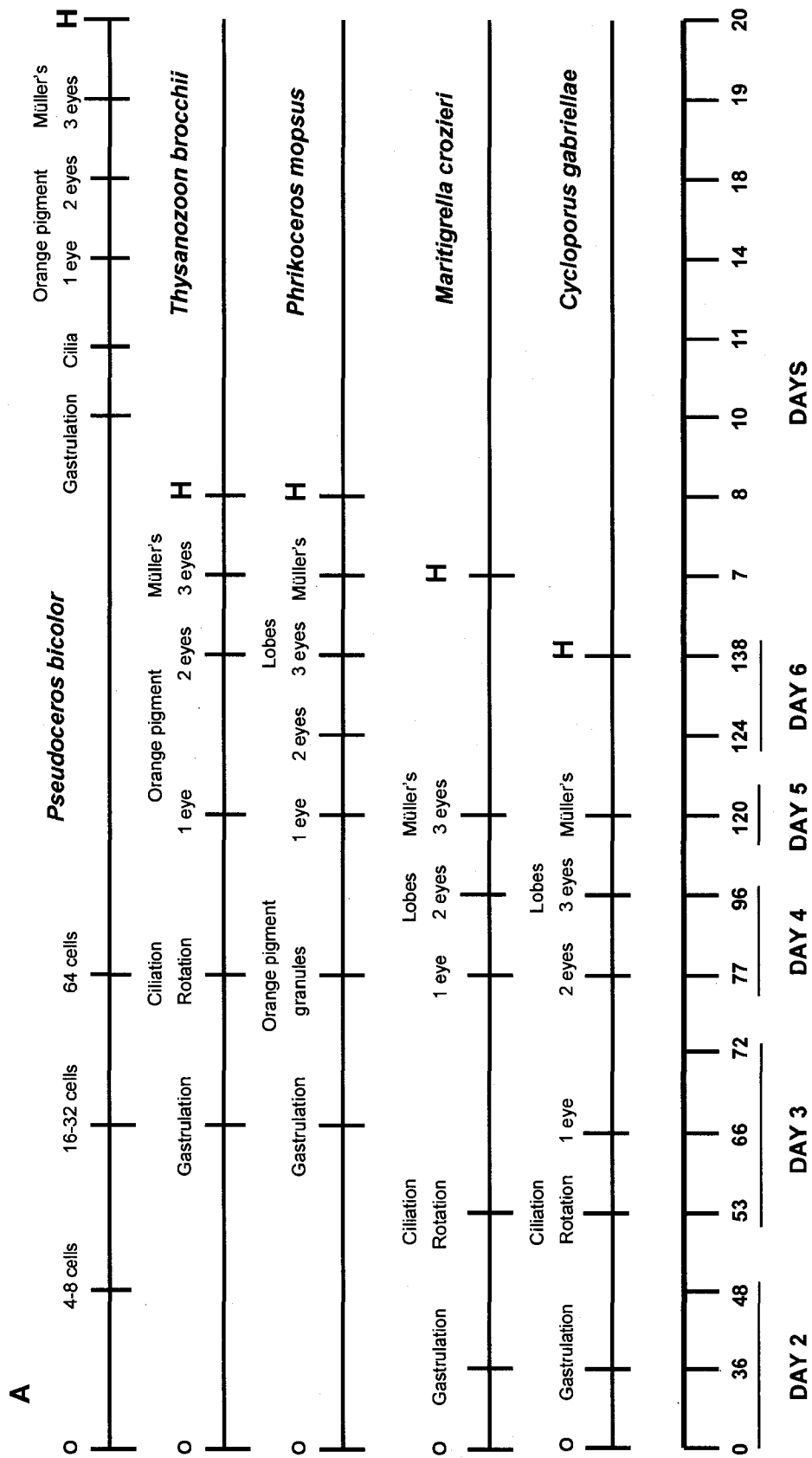
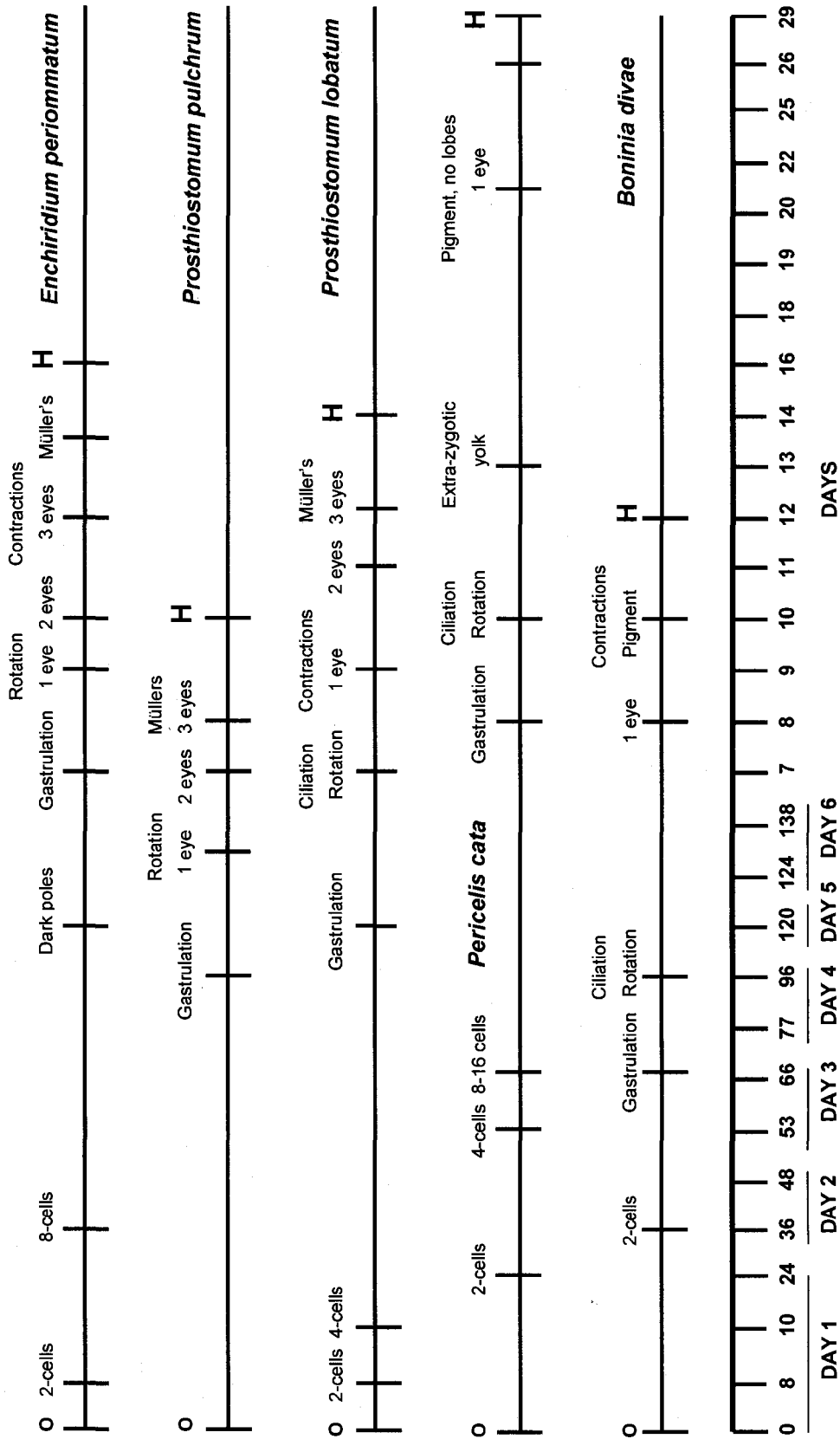


Figure 10. Comparative time line for the embryonic development of ten species of coryleans. (A) Time line from oviposition to hatching of Pseudocerotidae and Euryleptidae; (B) Time line from oviposition to hatching of Prosthiostomidae, *Pericelis cata*, and *Boninia divae*. O= oviposition, G= gastrulation, H= hatching.

Cont.

B



After oviposition, the zygotes were of an even white or cream color with a small darker area in the center that corresponded to the nucleus. Pseudocerotids, especially *Phrikoceros mopsus* and *Thysanozoon brocchi*, displayed a conspicuous black area along one edge of the eggs; this area eventually migrated to the animal pole (Figs. 9I-L).

Generally, embryonic development of cotyleans was similar for all species examined. There was some variation in the diameter of the eggs and egg capsules (Table 3). Cleavage followed the same pattern described for acotyleans, and the first divisions occurred almost immediately after oviposition (Figs. 9A-H). The first polar body is formed at the animal pole (Fig. 9G, H). Gastrulation by epiboly started as early as day 2 (in Euryleptidae) or as late as day 8 (*Pericelis cata*) (Fig. 10A, B). Micromeres migrated towards the vegetal pole over the large macromeres resulting in a ciliated stereogastrula (Figs. 11A-D). Yolk cells in the interior of embryos were smaller than those observed in acotyleans, allowing for easier observations of events taking place on the surface of the embryo. Slow incomplete rotational movements were evident. A single eye spot appeared while embryos were still rounded in shape; again euryleptids developed an eye spot as early as days 3-4 and *P. cata* as late as day 21 (Figs. 11E-G). This was followed by an increase in the number and length of the cilia, resulting in more rapid rotations. Concurrently, yolk was rapidly absorbed, slight invaginations of the epidermis hinted at the beginning of the larva shape, and a second eye spot appeared (Figs. 11F-H). In most species, the two-eyes stage lasted for a few hours, after which a third eye formed (Fig 11I). The ciliated lobes became more conspicuous and the shape of the Müller's larva was evident. Small, translucent yolk granules were still present in the interior of the embryos (Figs. 11J). Over the next few days, the larvae remained inside their egg capsules, slowly rotating and contracting their bodies. An accumulation of brownish pigment in the center of their bodies indicated the formation of the pharynx and mouth. (Figs. 11K, L).

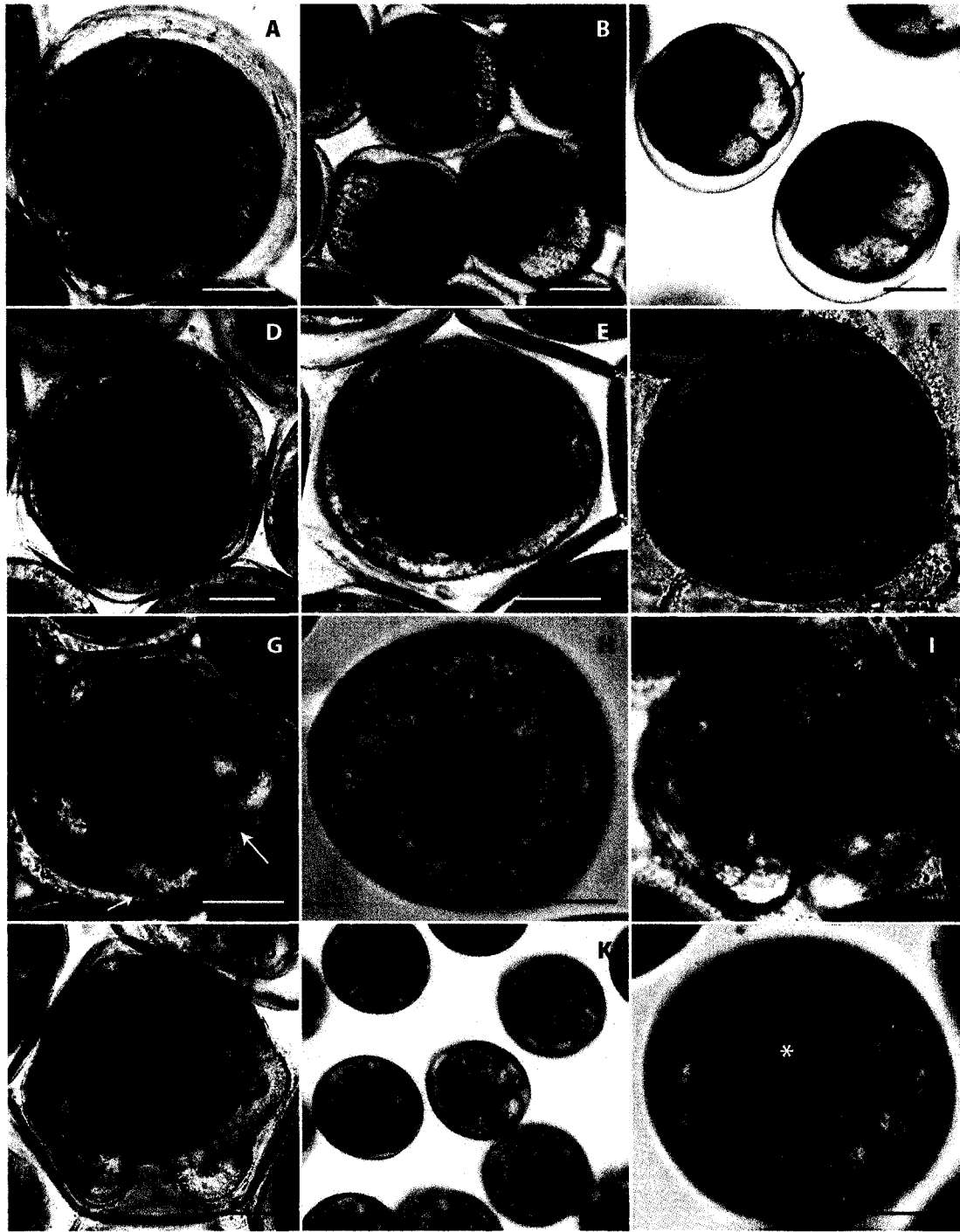


Figure 11. Embryonic development in cotyleans. (A, B) division of mesoblast; arrows indicate mesoblast cells; (C) gastrulation, dark area represent micromere migration towards animal pole; (D) stereogastrula; (E) formation of a single eye; (F, G) single-eye stage, arrows indicate invaginations of the epidermis forming the first lobes. Scale bars, 50 μm , (H) two-eyes stage with ciliated epidermis; lateral lobes are present; (I) ventral view of oral lobe (asterisk); (J) dorsal view of embryo with well-developed lobes. Scale bars, 25 μm , (K) Müller's larvae. Scale bar, 50 μm , (L) well-developed Müller's larva before hatching with three eyes and brownish pigment inside the body (asterisk). Scale bar, 25 μm . (A, D-G) *Pseudoceros bicolor*, (B, I, J) *Thysanozoon brocchii*; (C, H, K, L) *Cycloporus gabriellae*

Developmental timelines in cotyleans were more variable among species than for acotyleans. For example, within the Pseudocerotidae *P. mopsus* and *T. brocchii* shared the same time line for major developmental events and both hatched about one week after oviposition. Another pseudocerotid, *P. bicolor* on the other hand, did not gastrulate until day 10 and hatching did not occur until day 20 (Fig. 10A). Similarly, among Prosthiostomidae, *Enchiridium periommatum* hatched on day 16, *Prosthiostomum lobatum* on day 14, and *P. pulchrum* on day 10 (Fig. 10B). Not only did the developmental time line of *P. lobatum* and *P. pulchrum* differ, but these two congeners also showed remarkably differences in the number of eggs per capsule and in details of the egg capsule surfaces as mentioned before. The most rapid development was observed in the two euryleptids (Fig. 10A).

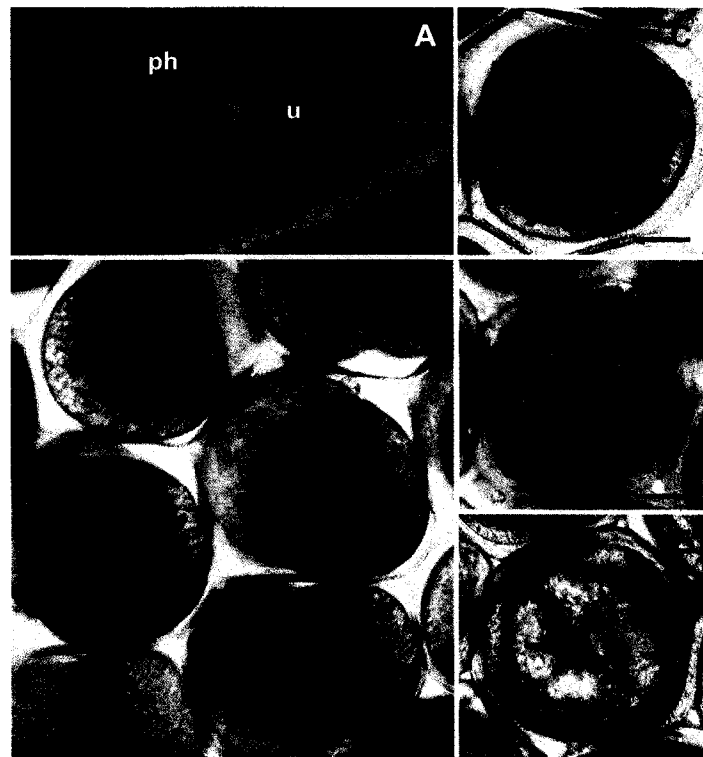


Figure 12. Pigmentation observed in pseudocerotids. (A) Ventral view of adult *Pseudoceros bicolor* with red pigment around uteri; p pharynx; u. uteri; (B) egg plate after oviposition containing red zygotes; (C) post-gastrulation embryo with original red coloration and orange pigment dispersed throughout the epidermis. Scale bars, 50 µm, D) pigment granules in *Phrikoceros mopsus* dispersed over the entire surface; (E) post-gastrulation embryo of *Thysanozoon brocchii* with orange pigment dispersed through the epidermis. Scale bars, 25 µm.

Unlike other species, the uteri of live adult female *P. bicolor* were intensely red (Fig. 12A). As a result, zygotes and embryos of this species exhibited a prominent red cytoplasmic coloration. However, the gelatinous covering of the egg plate and the egg capsules were of the same brownish color as in other species (Fig. 12B). Pigment granules were only observed in pseudocerotids (*P. bicolor*, *T. brocchii*, and *P. mopsus*). Large, orange pigment spots were noticeable shortly after gastrulation (Figs. 12C-E), which began to disperse all over the surface of the embryos and which persisted to the larval stage.

Variations in development were observed for *Pericelis cata* and *Boninia divae*. In *P. cata* large extra-zygotic yolk droplets surrounded the embryos after gastrulation. These droplets diminished the ability to detect any changes for the next 7 days. Furthermore, a case of poecilogony for *P. cata*, in which juveniles and larvae were released simultaneously from the same egg plate was documented (see Chapter III). However, the larvae of *P. cata* did exhibit a few morphological differences when compared to other cotyleans (see Chapter III). *Boninia divae*, a multiembryonic species (Fig. 4D; 16E), completed development in about 12 days. As embryos contracted within the egg capsules, their body forms changed to a more triangular shape. At hatching, larvae of *B. divae* appeared similar to those of *P. cata*. They hatched with a single eye spot, dorsal lobes that are extremely reduced, and only two very small, barely visible lateral lobes. It is possible that *P. cata* and *B. divae* represent cotyleans with Götte's rather than Müller's larvae typical of the suborder (embryology of *B. divae* is the subject of a separate manuscript).

Table 4. Comparison of time of development, developmental mode, and culture.

Species	Time to Hatching (d)	Developmental Mode	Culture Temperature (°C)
<i>Armatoplana lactoalba</i>	15	Direct	23
<i>Notocomplana lapunda</i>	19	Direct	23
<i>Melloplana ferruginea</i>	22	Direct	23
<i>Styloplanocera fasciata</i>	15	Direct	23
<i>Imogine zebra</i>	21	Direct	22
<i>Pleioplana atomata</i>	42	Direct	15
<i>Cycloporus gabriellae</i>	6	Indirect	23
<i>Maritigrella crozieri</i>	7	Indirect	23
<i>Pericelis cata</i>	±29	Indirect	23
<i>Boninia divae</i>	12	Indirect	23
<i>Pseudoceros bicolor</i>	±20	Indirect	23
<i>Phrikoceros mopsus</i>	8	Indirect	23
<i>Thysanozoon brocchii</i>	8	Indirect	23
<i>Enchiridium periommatum</i>	16	Indirect	23
<i>Prosthlostomum lobatum</i>	15	Indirect	23
<i>Prosthlostomum pulchrum</i>	10	Indirect	23

Hatchlings (Figs. 13A-D; 14A-J)

Juveniles: The newly hatched worms appeared as miniature adults with prominent dark eye clusters. The size of juveniles ranged from 192 μm (*Armatoplana lactoalba*) to 490 μm (*Pleioplana atomata*). The elongated bodies of juveniles were not completely flattened and had broad, rounded anterior and tapered posterior ends (Figs. 13A-D). The interior of their bodies contained brownish pigment that extended anteriorly along lateral branches. The mouth was located on the ventral median line and usually was sealed for several days post-hatching (Fig. 13A, C). The epidermis was completely ciliated (Fig. 13D), and juveniles were actively swimming using their cilia and exhibiting positive phototropism. For several days after hatching, the juveniles swam by ciliary action only

and muscular contractions were rarely observed. Juveniles had short but prominent sensory hairs at the anterior margin that were arranged in a species-specific pattern. For example, *Notocomplana lapunda* was characterized by two anterior sensory hairs, whereas *Melloplana ferruginea* had four (Figs. 13A, B). In general, juveniles also had two longer sensory hairs at their posterior ends (Figs. 13A, C). Settlement to the benthos occurred about eight days post-hatching in *M. ferruginea* and coincided with the opening of the mouth and a flattening of the body.

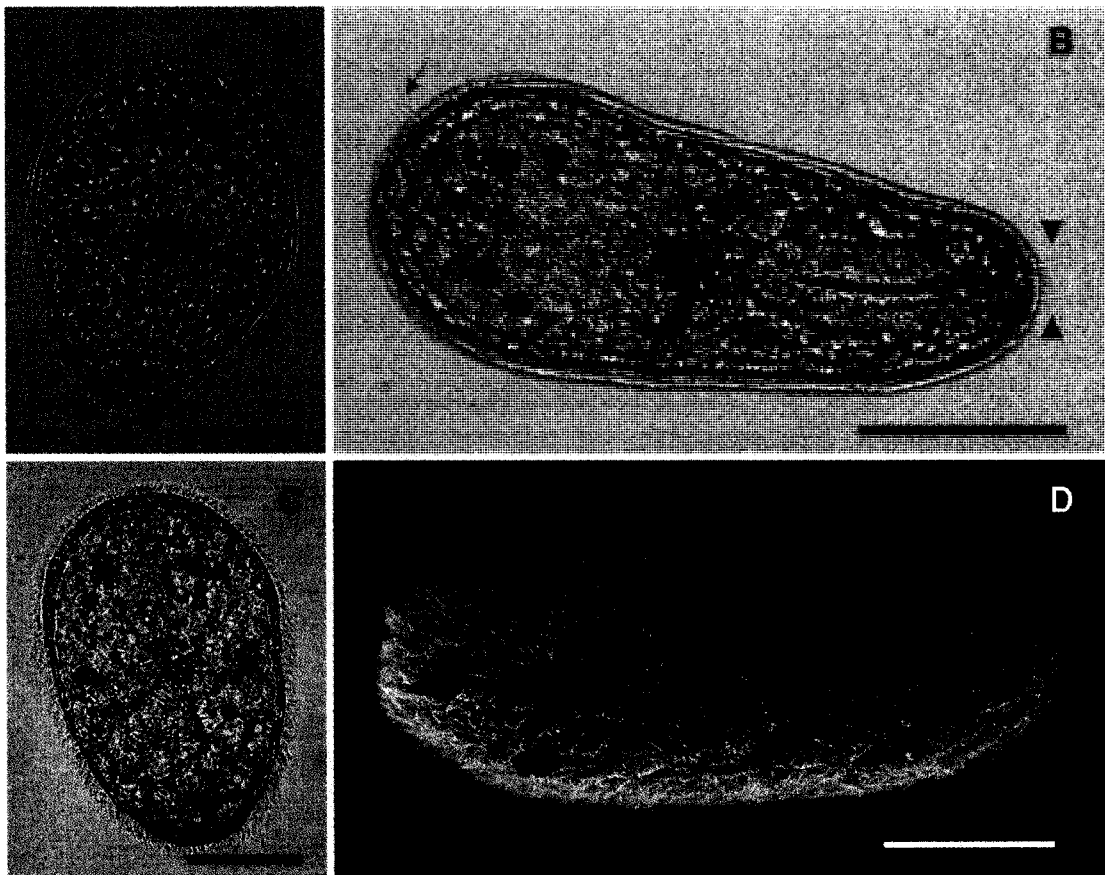


Figure 13. Juveniles of acotylean polyclads. (A, B) Newly hatched juveniles of *Melloplana ferruginea* showing an elongated shape, clusters of eyes, pigment, closed mouth, and ciliated epidermis, arrows indicate apical cilia; arrowheads show posterior cilia; (C) newly hatched juvenile of *Notocomplana lapunda* with two apical cilia; (D) Scanning electron micrograph of a juvenile of *M. ferruginea* showing the entire ciliated body surface. Scale bars, 50 μ m.

Larvae: Müller's larvae swam vigorously in the water column exhibiting positive phototropism. Larvae hatched with three eyes, and eight ciliated lobes (Figs. 14A, B)

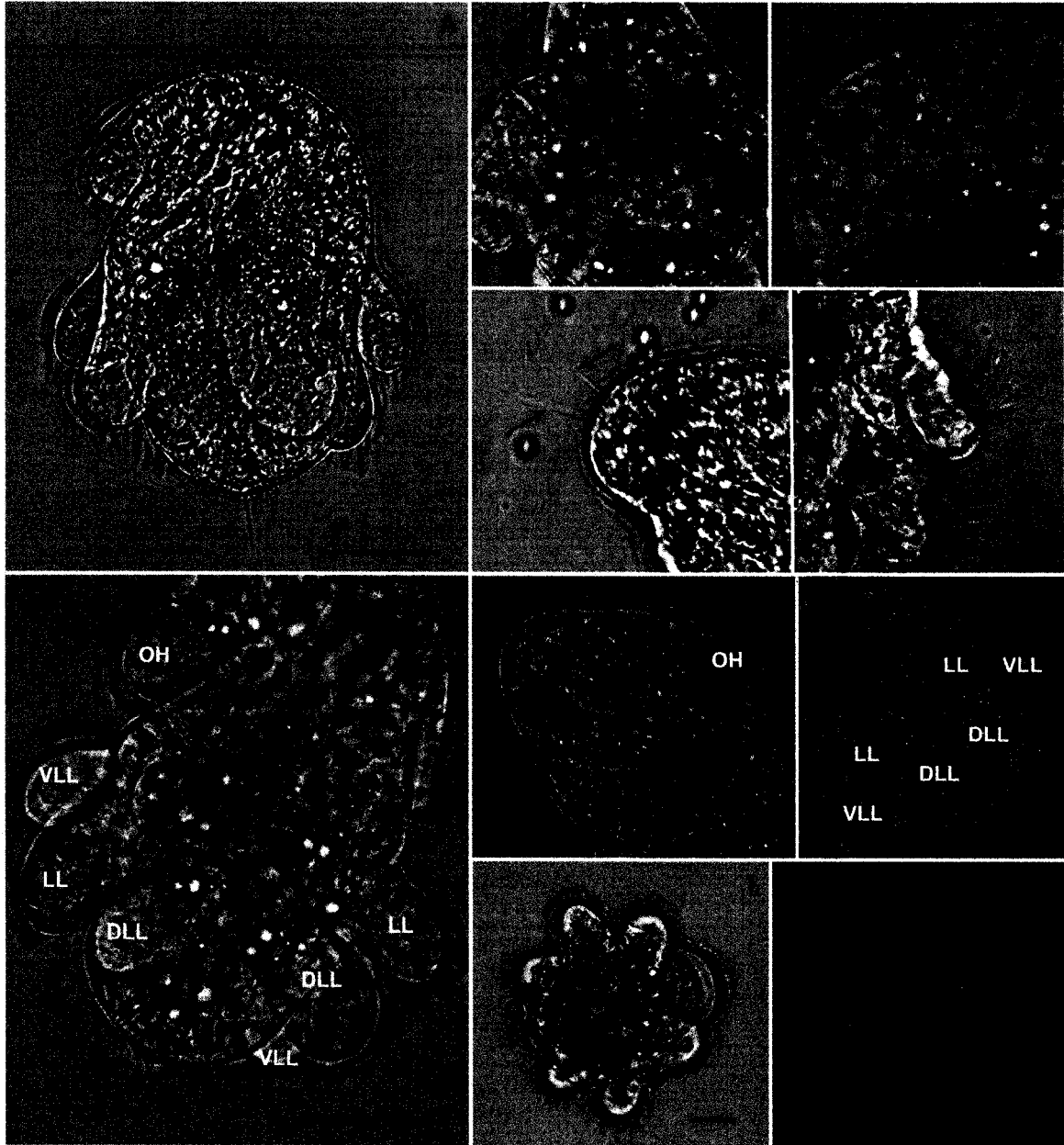


Figure 14. Müller's larvae of *Cycloporus gabriellae*. (A, B) Newly hatched larva with three eyes and eight ciliated lobes; arrows indicate apical and posterior tufts. Scale bars, 50 μm ; (C) circular mouth, (D) rhabdites of the epidermis (arrows), (E) detail of apical tuft (arrows), (F) detail of lateral lobes showing long cilia, (G) detail of oral lobe, (H) detail of a pair of dorsolateral lobes, (I) larva of circular shape showing eight ciliated lobes. Scale bars, 20 μm ; (J) Müller's larvae of *Enchirdium periommatum* exhibiting a square shape. Scale bar, 25 μm . OH, oral hood; VLL, ventro-lateral lobes; DLL, dorsolateral lobes; LL, lateral lobes.

and measured about 150-175 μm . The mouth opening was transparent and surrounded by concentrated dark pigment that appeared just prior to hatching (Fig. 14C). Rudimentary rhabdite bundles were formed in the epidermis (Fig. 14D). The anterior end

was characterized by an apical tuft consisting of two long sensory hairs (Fig. 14E). At the posterior end, a single long sensory cilium was visible (Fig. 14A). Locomotion was by means of cilia, especially those of the lateral lobes (Fig. 14F).

Eight lobes were distributed evenly around the body. It was possible to distinguish one broad, mid-ventral lobe, forming the oral hood (OL), a smaller mid-dorsal lobe (MDL), paired ventrolateral lobes (VLL), a pair of dorsolateral lobes (DLL), and two lateral lobes (LL) (Figs. 14B, G-I). The shape of the prosthiostomid Müller's larvae was more square-like anteriorly and the pharynx was elongate with a tubular shape (Fig. 14J).

Feeding and Metamorphosis (Figs. 15A-F)

Immediately after hatching, larvae of *Cycloporus gabriellae* were fed with the microalga *Isochrysis* sp. The size of the microalgae was 6.25 μm and the larval mouth opening was 27.5 μm (Figs. 14A, 15A). About 12 hours later, all larvae contained high concentrations of green algae in their bodies and around their mouths (Fig. 15B). Larvae were maintained in the laboratory for an additional 20 days. During that time, few metamorphic changes were observed. On day 14, mid-dorsal and dorsolateral lobes were slightly reduced (Fig. 15C), and two days later, larvae had reduced their overall size and their mid-dorsal lobes had completely disappeared (Figs. 15D, E). On day 20, all evidence of lobes had disappeared and the larvae began to elongate (Fig. 15F). Over the next few days, all metamorphosing larvae died.

Prosthiostomum lobatum and *P. pulchrum* were maintained in the laboratory without food. They began settlement and reabsorbing lobes 15 and 11 days post-hatching, respectively. *Phrikoceros mopsus* began metamorphosis 6 days after hatching. Species maintained without food decreased their overall size considerably and once

their lobes were reabsorbed, their bodies became more rounded, rather than elongate. Unfortunately, complete metamorphosis was never observed.

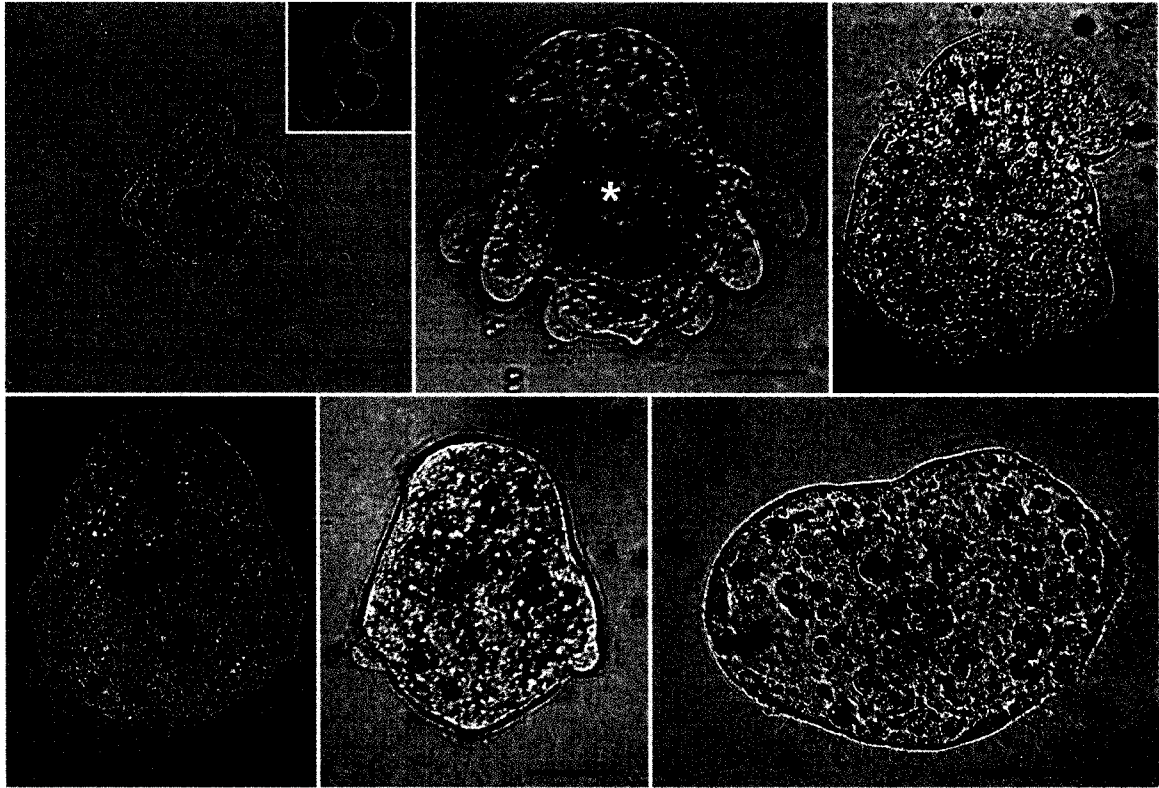


Figure 15. Metamorphosis of *Cycloporus gabriellae*. (A) Swimming larva with microalgae post-hatching; inset: detail of microalgae *Isochrysis* sp., Scale bar, 5 μ m; (B) larva with evidence of microalgae in the interior of the body seen as green pigment (asterisk), (C) 14-day larva with reduced dorsal lobes, (D) 16-day larva with complete reduction of dorsal lobes, (E) 16-day larva reduced in size and with only small lateral lobes. Scale bars, 50 μ m; (F) 20-day larva without lobes and elongated body. Scale bar, 25 μ m.

Discussion

The results of this study show that there are considerable differences as to egg plates, egg capsule morphology, size and number of eggs, and developmental time lines among 16 species of polyclad flatworms. Different types of egg plates were identified which are consistent with previous descriptions (Selenka 1881; Lang 1884; Kato 1940; Anderson 1977; Ishida & Teshirogi 1986). To date, no categorization of the shape of egg plates exists. In the following, a descriptive system is proposed that may facilitate future

comparisons. However, because it is not possible to correlate egg plate type with existing classification systems, these categories are purely descriptive. Based on the results of this study and on information from the literature (Selenka 1881; Lang 1884; Kato 1940; Anderson 1977) the following descriptive categories can be recognized: a) irregular-shaped disc, b) irregular-shaped, bilayered disc, c) unordered egg batches, d) single spiral, e) double spiral, f) zig-zag single/double row, and g) straight single/double row. I recognize that the type and texture of the substrate may have a strong influence on the shape of deposited egg plates. Furthermore, it is likely that the shape of an egg plate is based mostly on a parental strategy to protect the developing embryos against external factors rather than being of systematic significance.

Formation of egg capsules has been examined at the ultrastructural level for a few species of polyclads (Boyer 1972; Domenici et al. 1975; Ishida et al. 1981; Ishida & Teshirogi 1986), although their morphologies and the functions have received little attention. Egg capsules are extracellular material surrounding the eggs and are important for the development and survival of the embryos. Egg capsules, in conjunction with the gelatinous material that forms the egg plates, provide protection from external factors and maintain a favorable environment for the continued development of the embryos (Jägersten 1972; Davis 1968). The results of this study shed some light on the importance of egg capsule morphology in the development of polyclad embryos. Acotylean egg capsule morphology was uniform, ornamented by a network of circular ridges and irregular sculpturing. Similar morphologies have been described for *Planocera multitentaculata*, *Pseudostylochus* sp. and *Notoplana humilis* (see, Kato 1940; Ishida & Teshirogi 1986). The acotyleans examined were all direct developing species with large-diameter eggs and developmental times of well over two weeks. Such time periods translate into a need for long-term protection. Hence, the morphology observed here may represent the optimal design for long-term strength without

sacrificing the ability to break at hatching. Ishida & Teshirogi (1986) show that egg capsules of two acotyleans break in predictable ways along the boundary areas between the circular shield-like plates. These authors attribute this to sclerotin-containing proteins in the circular plates that impart structural support to the capsules.

Egg capsule morphologies among cotyleans showed greater variation. Euryleptid embryos, which hatch in 6-7 days, were protected by thin delicate egg capsules with a smooth surface and lacking opercula. Similarly, pseudocerotid capsules of rapidly developing species (8 days) were homogeneously smooth and thin-walled and equipped with opercula. These results suggest that there is a correlation between short developmental times and delicate egg capsules. Surface textures of the egg capsules of the remaining cotyleans appeared rough and in some cases, covered by a fibrillar network (e. g., Prothiostomidae). Unlike egg capsules of Acotylea and Euryleptidae, these capsules did not fragment at hatching. Only the opercula were broken which suggests that these capsules are well sclerotized (Ishida & Teshirogi 1986). Species like *Pericelis cata* and *Enchiridium periommatum* had the thickest egg capsules, a fact that is directly correlated with the longest developmental times.

Hatching is achieved by mechanical action. Embryos were observed escaping their capsules by repeatedly contracting their bodies and by rapid rotational movements mediated by cilia. *Melloplana ferruginea* and *Pleioplana atomata* pushed against the walls of the capsules until they ruptured. Egg capsules were somewhat elastic and the walls deformed as juveniles elongated and enlarged their bodies (Fig. 8H). It is possible that breaking the egg capsule is facilitated by the release of enzymes as has been shown for the proseriate, *Monocelis fusca* (Giesa 1966). From unsuccessful fluorescent staining attempts during this study, I know that polyclad egg capsules are highly impermeable. Hence, hatching is probably not mediated by an increased osmotic pressure.

Finally, egg capsules protect the embryos against bacterial infections and attacks by Protozoa (pers. observ). Acotyleans with long developmental times (*Pleioplana atomata*, *Imogine zebra*) never exceeded the 32-cell stage when their egg capsules had been removed. Indirect developing species with short embryonic time lines (i.e. *Phrikoceros mopsus*, *Thysanozoon brocchii*, and *Cycloporus gabriellae*) on the other hand, were able to complete their development even in the absence of egg capsules (pers. observ.), demonstrating that encapsulation is obligatory for the early development of species with long developmental time lines.

The general surface morphology of the egg capsules is useful for the differentiation of some higher taxonomic levels. For example, members of the suborder Acotylea and the family Pseudocerotidae have characteristic ornamentations that allow for initial taxonomic placement of field-collected egg capsules. For other polyclad taxa though, similar capsules may be produced by taxonomically diverse species (e. g., *Pericelis cata*, *Boninia divae*). Differentiation to the specific level using egg capsule morphologies clearly is not possible due to insufficient variation (e. g., the two species of *Prosthlostomum*).

Egg size has been correlated with developmental time, where larger eggs develop more slowly than smaller eggs (Perron 1981; Marshall & Bolton 2007). Comparing egg sizes of the 16 species, it was possible to link larger eggs to direct developing species and longer developmental time and most but not all, indirect developing species to smaller egg sizes. Although this general trend was seen for some of the species, the correlation coefficient suggested that no significant correlation exists between the two variables. Cleavage is influenced and modified by the amount of yolk present (Hyman 1951; Kato 1940). Larger and yolky eggs were characterized by extended periods of time inside the egg capsules, a trend clearly observed in acotyleans and in *Pericelis cata* (Chapter III).

The number of eggs per egg capsule does not appear to be of systematic value. Monoembryonic egg capsules were the rule for Acotylea with one exception. Some capsules of *Pleiolana atomata* contained two embryos. However, it is likely that this represents a developmental aberration because twins usually hatched as conjoined juveniles, which most likely cannot survive. Kato (1940) described multiembryony for one other acotylean species, *Planocera reticulata*, a species characterized by other unusual developmental features, adelophophagy and intracapsular development. The number of embryos was highly variable among cotyleans, ranging from 2 to 6 eggs even within the same species (see Table 3, Figs. 16A-F).

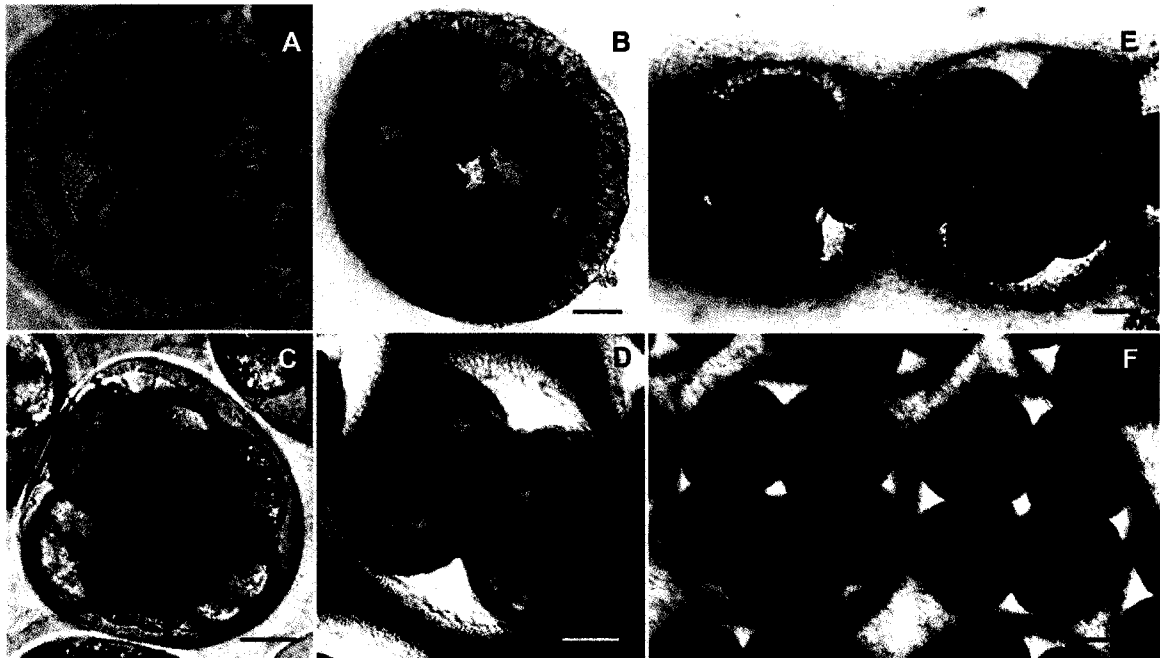


Figure 16. Variation in number of eggs per capsule in cotyleans. (A) Two encapsulated eggs in *Prosthlostomum lobatum* during gastrulation; (B) four encapsulated eggs in *P. lobatum*; (C) six eggs per capsule in *Prosthlostomum pulchrum*; (D) two eggs per capsule in *Enchiridium periommatum*; (E) five and six eggs per capsule in *Boninia divae*; (F) four and five eggs per capsule in *Pericelis cata*. Scale bars, 50 μ m.

I propose that rather than being a species-specific feature, zygote encapsulation is more related to female body size and the general structure of the female reproductive system. The female system in polyclads includes paired ovaries and oviducts, a vagina,

an antrum leading to a gonopore, cement glands, and sometimes uterine vesicles (Hyman 1951; Faubel 1983, 1984; Prudhoe 1985). Lang's vesicle is a structure found only in Acotylea and the Boniniidae, whereas cement pouches characterize cotyleans. In general, acotyleans have a strongly muscular, curved, and long vagina. The vagina of Cotylea on the other hand, is a weakly muscularized, relatively straight, and short organ. I hypothesize that eggs passing through the curved vagina of acotyleans have to move in single file because of the rigidity of its muscular wall, which does not allow for great expansion. As eggs pass through the female system, they are individually surrounded by secretions from egg shell glands thus, forming mono-embryonic egg capsules.

In cotyleans, multiple eggs can migrate through the female system because the vaginal wall is not strongly muscular. Two cotylean families that do have fairly well muscularized vaginal walls are the Pseudocerotidae and Euryleptidae, and in fact they produce egg capsules containing single embryos. In Cotylea, cement glands are well developed and usually contain a pair of cement pouches. I contend that mature eggs are collected in the chamber-like cement pouches which act as reservoirs from which multiple eggs are surrounded by secretions from the cement glands and eventually are deposited into a single egg capsule. Pseudocerotids are characterized by small cement pouches that are not well-developed, and hence they produce mono-embryonic capsules. Therefore, it is likely that the musculature of the vaginal wall, the structure of the vagina itself, and the presence of well-developed cement pouches and cement glands are the driving factors in determining the number of embryos per egg capsule. An examination of the number of zygotes per egg capsule in other species will ultimately determine if this hypothesis proves correct.

ORIGINAL CONTRIBUTION BY Ph.D CANDIDATE IN CHAPTER TWO INCLUDES
THE DESCRIPTION OF EGG LAYING, DEVELOPMENTAL TIME LINES, AND
EMBRYONIC STAGES

CHAPTER II

REPRODUCTION, DEVELOPMENT, AND PARENTAL CARE IN TWO DIRECT-DEVELOPING FLATWORMS (PLATYHELMINTHES: POLYCLADIDA: ACOTYLEA)¹

Introduction

Polyclad reproduction and development have been the focus of numerous studies dating to the earliest investigations of polar body formation, fertilization and cleavage patterns (Hallez 1879; Selenka 1881; Lang 1884; Götte 1878, 1882; Surface 1907). Since then, different aspects of reproductive behavior, embryogenesis, and hatching have been recorded for a number of polyclad species (Kato 1940; Christensen 1971; Lytwyn & McDermott 1976; Anderson 1977; Ballarin & Galleni 1984). Today, polyclads are models for understanding spiralian development (Boyer & Henry 1995; Henry et al. 1995), and are used in studies of cell lineage tracing using fluorescent and immunohistochemical approaches (Boyer et al. 1996, 1998; Younossi-Hartenstein & Hartenstein 2000).

As is true for other flatworms, polyclads are simultaneous hermaphrodites, with concurrently functional male and female reproductive structures. Therefore, insemination can potentially be reciprocal with each partner contributing and receiving sperm via direct sperm transfer. Alternatively, insemination may be unilateral where one animal

¹ Rawlinson K., D.M. Bolaños, M.K. Liana, M.K. Litvaitis. Reproduction, Development, and Parental Care in two Polyclad Flatworms (Platyhelminthes: Acotylea). *Journal of Natural History (In press)*.

performs as the male and the partner animal acts as the female. Unilateral insemination is achieved by indirect sperm transfer, which involves either hypodermal insemination or dermal impregnation. During hypodermal insemination, one individual injects sperm anywhere through the epidermis of the partner animal, using an armed penis (Hyman, 1951). In dermal impregnation, spermatophores are deposited by the male copulatory organ of one individual onto the dorsal surface of the partner. Deposited sperm are absorbed through the epidermis and then move through the parenchyma to the eggs (Hyman 1951; Galleni & Gremigni 1989). The female gonopore is used only during the process of egg laying.

In many simultaneous hermaphrodites reciprocity of sperm exchange is the rule. Pre-copulatory behavior of reciprocally inseminating individuals has been studied extensively in triclad flatworms (Vreys & Michiels 1995, 1997; Vreys et al, 1997) and in the microturbellarian *Macrostomum* sp. (Schärer et al. 2004). Hyman (1951) contends that copulatory behavior among all free-living, reciprocally inseminating flatworms is similar. Generally, copulation involves pressing the genital regions together, commonly elevating them in the process and a mutual insertion of the copulatory structures into the respective female gonopores. The duration of copulation is highly variable within the group, ranging from minutes to hours (Hyman 1951; Kato 1940). In polyclads with true copulation, sperm is deposited through the female gonopore and probably stored in Lang's vesicle. From there, sperm moves either into the oviducts or the uteri to fertilize the eggs. True copulation has been linked to the presence of a Lang's vesicle in the female reproductive system (Galleni & Gremigni 1989).

Parental care in which adults cover laid egg masses with their bodies, has been shown in *Echinoplana celerrima* Haswell, 1907 (Lee 2006), and in many stylochids (Pearse & Wharton 1938; Rzhepishevskij 1979; Galleni et al. 1980; Murina et al. 1995;

Merory & Newman 2005), and may represent a form of guarding against potential predators. However, hatching success was not dependent on time spent guarding the eggs even in the presence of putative flatworm predators (Lee 2006). Furthermore, at least two planoceric polyclads are known to secrete tetrodotoxin into their eggs (Miyazawa et al. 1986; Tanu et al. 2004), affording potential protection against predation. Therefore, the function of covering laid egg masses by adults may be multifaceted and may differ among species.

The major goals of this chapter were threefold. First, we describe the reproductive behavior, egg laying, and developmental time line of the acotyleans *Pleiolana atomata* (Müller OF, 1776), and *Imogine zebra* (Verrill, 1882). Second, we also examine potential correlations between the size of the parent animal and the number of eggs and egg batches laid. Finally, we assess the effect of parental care on egg hatching rate in *P. atomata* and *I. zebra* and discuss possible functions of this behavior.

Materials and Methods

Specimen Collection

Pleiolana atomata was collected from under rocks in the intertidal and shallow subtidal zones in March 2006 and 2007 at Odiorne Point, New Hampshire, USA (43° 02.395' N; 70° 42.899' W). Many specimens were found hiding under clusters of the green sea urchin *Strongylocentrotus droebachiensis* (Müller OF, 1776). Mature specimens were identified by the presence of sperm in the sperm ducts visible by eye. *Imogine zebra* was obtained from the Marine Biological Laboratory (Wood's Hole, Massachusetts). The specimens had been collected from gastropod shells that were inhabited by the hermit crab, *Pagurus pollicaris* Say, 1817. Measurements of adult body

length (in mm) were taken from live animals when they were in a relaxed and quiescent state.

Reproductive Behavior

Pleiolana atomata and *Imogine zebra* adults were kept at 15°C in constant darkness; they were not fed, and the seawater was changed every 5 days. After an acclimation time of 2 days, similar-sized, mature specimens of *P. atomata* were placed in Petri dishes and reproductive behavior was recorded. *I. zebra* were not acclimated, instead animals were placed together immediately. Pre-copulatory and copulatory behaviors were recorded digitally as iMovies, using a Canon XL1s. Mode of insemination was noted, as well as length of time of copulations (in min).

Reproductive Effort and Embryonic Development

After recording reproductive behavior, individual specimens of both species were isolated into separate plastic bags. Over the course of 6 weeks, estimators of reproductive effort based on the number of batches of eggs and the number of eggs per batch were obtained for each individual flatworm.

The animals deposited eggs on the sides of the plastic bags, which could then be examined and counted under a Nikon SMZ-U stereomicroscope. To document embryonic development, photographs were taken every 24 hours using a Nikon CoolPix 990 digital camera attached to the stereomicroscope. Embryos of *Pleiolana atomata* were kept in plastic bags at 15°C during their development, whereas embryos of *Imogine zebra* were kept in Petri dishes at 22°C in the laboratory under natural light cycles, and water was changed 3 times a week.

Parental Care

The influence of parental care on hatching success was determined by calculating the percentage of eggs in each batch that developed to hatching stage in the presence or absence of a parent. Two approaches were used to assess the effects of parental care on hatching success. In the first approach (Treatment 1), 14 adult individuals of *Pleioplane atomata* and 6 adult individuals of *Imogine zebra* were divided into two equal groups. In one group, adults remained with their egg batches over the developmental period but in the second group, every egg batch laid was removed and reared in the absence of adults. The second approach was designed to exclude the effects of individual variability of fecundity (Treatment 2). Eight adult individuals of *P. atomata* and three adult specimens of *I. zebra* were used. In this case, the first three egg batches laid were removed and reared in the absence of the parent. The next three egg batches laid by the individual were kept in the plastic bag with the adult and used in the parental care treatment.

A possible variable that could confound the results of Treatment 2 is whether egg viability decreases over time with every batch laid. Data from Treatment 1 provided a time series of egg batches from many individuals, allowing us to test egg viability over time. The first 6 egg batches were divided into early (batches 1-3) and late (batches 4-6) groups. Twelve individuals of *P. atomata* and 8 individuals of *I. zebra* laid over 6 batches each and their hatching rate was analyzed.

Data Analysis

For both species, the number of eggs and batches laid was plotted against adult body length and the relationship and significance between them was evaluated using pairwise Spearman rank correlations (r_s). Analysis of variance (ANOVA) was used to test hypotheses about the effects of parental care on the percentage-hatching rate of egg

batches. All data were arcsine transformed. Data of Treatment 1 were analyzed using a one-way ANOVA with 'parental care' as a fixed factor. Because the number of batches per individual varied, the variation in hatching success was analyzed for a subset of batches: 4 per *P. atomata* adult and 6 per *I. zebra* adult. Treatment 2 data were analyzed by a two-way nested ANOVA (fixed factor = parental care, random factor = individual). A one-way ANOVA was used to assess variation in egg viability (hatching rate) between early- and later-laid batches. All tests were carried out using Minitab 13.30. Homogeneity of variance was tested using Levene's test.

Results and Discussion

Reproductive Behavior

Prior to copulation, *Pleiolana atomata* individuals glided over and around each other (Fig. 17A). After raising their caudal ends, both individuals prodded the ventral surface of partners with an everted penis (Fig. 17B-C). This may represent attempts at finding the female gonopore. The animals copulated (Fig. 17D), and transferred sperm directly into the female gonopore. Sperm transfer is assumed to be reciprocal. Copulation lasted for 18.2 ± 5.3 minutes (mean \pm s.d.) (maximum 49 minutes; $n = 10, 8$ pairs).

Sexually interacting individuals of *Imogine zebra* glided over and around each other (Fig. 18A), raised their caudal ends repeatedly (tail twitching), everted their penes, and prodded the dorsal surface of their partners (Fig. 18B, C). Usually just one individual undertook this behavior but occasionally, prodding occurred reciprocally. Alternatively, many individuals became involved, in which case one individual received sperm and at the same time, donated sperm to a third worm (Fig. 18D). Mode of insemination was by dermal impregnation (Fig. 18C) and pairs of worms took turns to donate and receive

sperm. Spermatophores up to 500 μm in size were deposited onto the epidermis (Fig. 18E). Mean length of time leading to sperm transfer was 8.2 ± 5.6 minutes (mean \pm s.d.) ($n = 10$, 8 pairs).

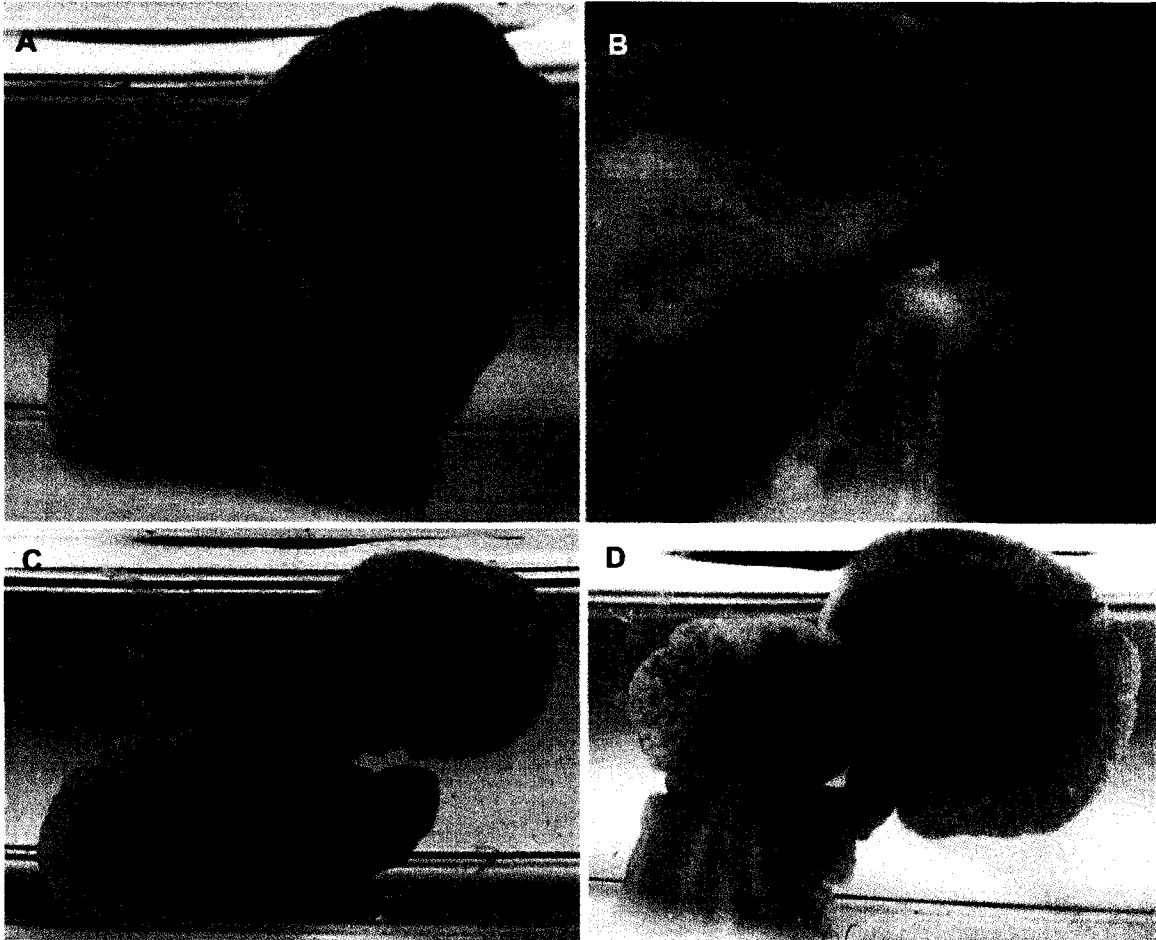


Figure 17. *Pleioplana atomata* reproductive behavior: (A) initial encounters, (B) and (C) reciprocal prodding of ventral surface, and (D) copulation. Images are video stills.

Hypodermic insemination and dermal impregnation allow hermaphrodites to skew sexual interactions in favor of sperm donation instead of the presumably more costly sperm receipt. The role of each worm may be determined by precopulatory behavior (Michiels & Newman 1998). Precopulatory behavior involving violent bouts of penis fencing that usually leads to unilateral insemination has been reported for pseudocerotid polyclads (Michiels & Newman 1998). Among individuals of *Imogine*

zebra, dermal impregnation was less violent and consequently, resulted in less physical damage. Sperm donation, although not simultaneous, was generally reciprocal. Worms mated with many individuals and there appeared to be little discrimination in mate choice.

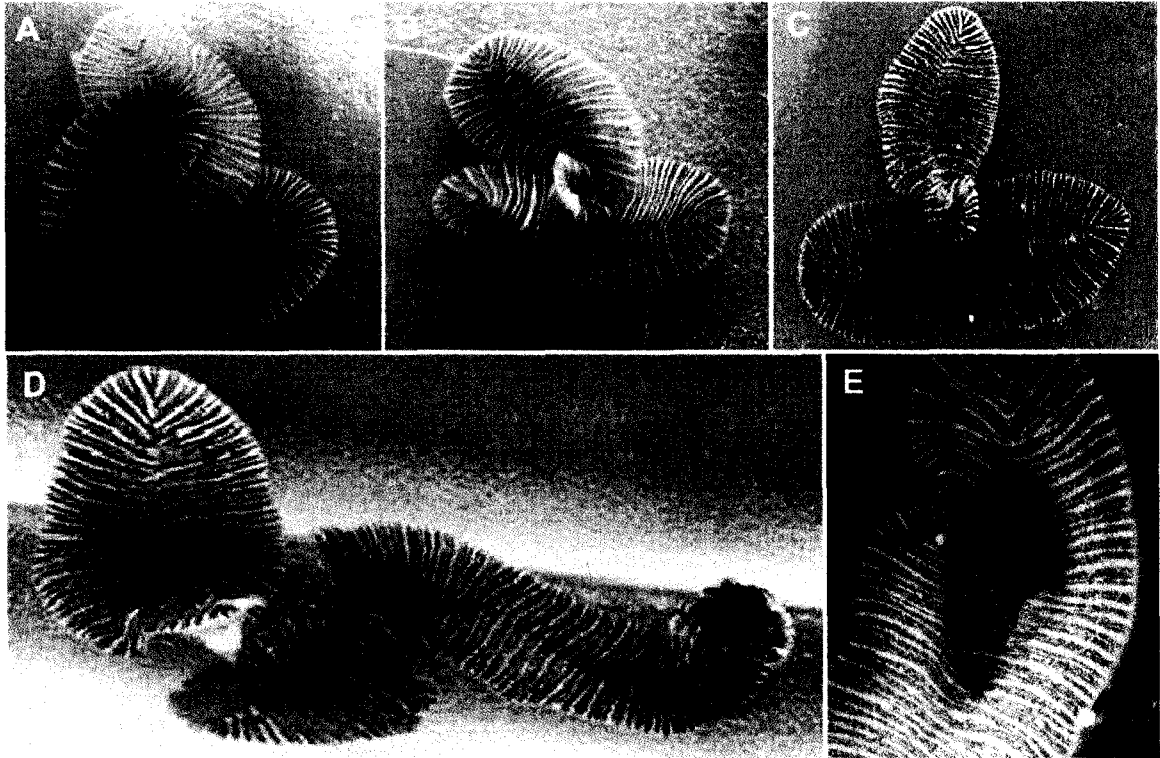


Figure 18. *Imogine zebra* reproductive behavior: (A) initial encounter and gliding over each other, (B) prodding of dorsal surface, (C) unilateral sperm transfer, (D) sperm transfer among multiple animals, (E) spermatophores on dorsal surface. Images are video stills.

Direct sperm transfer is thought to be the rule among polyclads that possess a well-developed female tract with a Lang's vesicle (Galleni & Gremigni 1989; Table 5). The function of Lang's vesicle is unknown. Bock (1913) considered it to be a secretory organ involved in the digestion of excess sperm and prostatic secretions. This function is supported by the fact that in some polyclads, the female reproductive tract is connected to the intestine (Prudhoe 1985). Kato (1940) on the other hand, proposed that Lang's vesicle is a sperm storage organ from which eggs will be fertilized at a later time. The two proposed functions are not mutually exclusive. In fact, it is possible that Lang's

vesicle initially functions as a storage organ for sperm received during copulation, followed by a digestive role in the removal of excess and aging sperm. Hence, species possessing a Lang's vesicle will assure sperm entry into the vesicle via true copulation (Galleni & Gremigini 1989). Species lacking a well-developed Lang's vesicle, on the other hand, will use indirect sperm transfer.

Although our observations on *Pleioplana atomata* and *Imogine zebra* support these hypotheses, the connection between mode of insemination and the presence/absence of a Lang's vesicle is not as clear-cut for other species (Table 5). Kato (1940) for example, recorded true copulation in species lacking Lang's vesicle (Table 5). On the other hand, it does seem unlikely that species using indirect sperm transfer would possess a Lang's vesicle, if it functions in sperm storage and digestion. More detailed observations of copulatory behavior in diverse species are needed to draw conclusions between reproductive system form and function.

Adult Size and Reproductive Effort

Egg masses of *Pleioplana atomata* and *Imogine zebra* were irregular in shape (Figs. 19A-B). The eggs of *P. atomata* were less regularly spaced, occasionally positioned on top of each other, and covered with a thick, gelatinous coating. Egg plates of *I. zebra* consisted of a single layer of closely packed and regularly spaced egg capsules. In both species, egg capsules consisted of transparent membranes that enclose round, cream-colored, entolecithal eggs. Generally, there was one egg per capsule, although occasionally two eggs per capsule were found in *P. atomata* (Fig. 19C). Most of these developed into two conjoined juveniles that adhered to each other even after hatching (Fig. 19D).

Differences in the shape of egg batches may be due to different habitat conditions of the respective species. Egg masses of *Pleioplana atomata* are attached

firmly to the substrate with a thick adhesive, possibly because the species inhabits the wave-exposed temperate littoral and sub-littoral zones. The more delicate, thinly coated egg plate of *Imogine zebra* on the other hand, is deposited within the body whorl of gastropod shells (Lytwyn & McDermott 1976) and therefore, may require less protection from the mechanical disturbances of the environment.

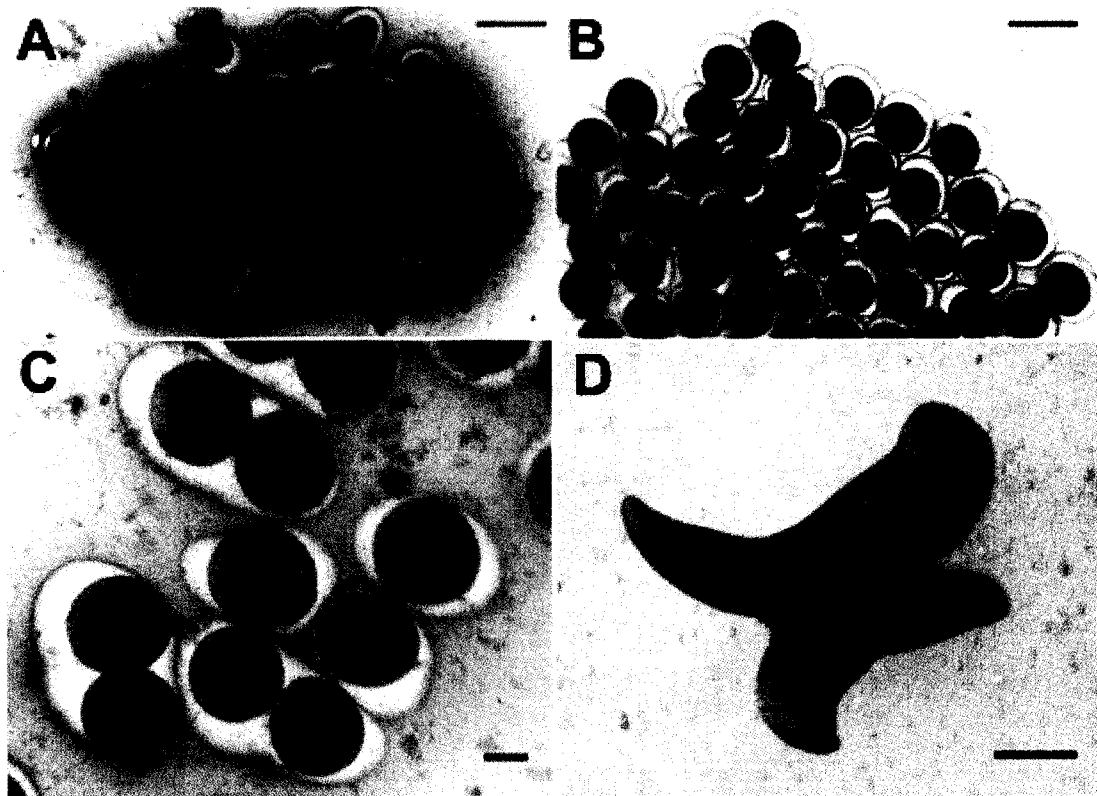


Figure 19. Egg masses: (A) *Pleioplana atomata*, (B) *Imogine zebra*. Scale bars, 500 μ m. *Pleioplana atomata*: (C) two eggs per egg capsule scale bar 150 μ m (D) conjoined hatchlings, scale bar 100 μ m.

Mature *Pleioplana atomata* individuals ranged in length from 8 to 20 mm (mean \pm s.d. = 13.7 ± 3.46 mm; $n = 43$). Egg diameter measured from 290 to 480 μ m (mean \pm s.d. = 385 ± 55.9 μ m; $n = 10$). Eggs within a batch numbered from 6 to 144, and 4 – 15 batches were produced over a 6-week period. An animal of 18 mm laid the highest number of eggs with 750 eggs over 9 batches. The number of eggs produced showed a significant positive relationship with animal length ($r_s = 0.52$, $P = 0.05$, $n = 15$) (Fig. 20A),

as did the number of egg batches ($r_s = 0.54$, $P < 0.05$, $n = 15$) (Fig 20B). Furthermore, there was a positive correlation between the number of eggs and number of egg batches ($r_s = 0.54$, $P < 0.04$, $n = 15$).

Mature *Imogine zebra* individuals ranged in length from 12 to 28 mm (mean \pm s.d. = 17.6 ± 4.3 mm; $n = 9$). Egg diameter measured 175 – 225 μm (mean \pm s.d. = 203 ± 14.8 μm ; $n = 14$). Eggs within a batch numbered from 10 to 490, and 6 – 17 batches were produced in 12 days. An animal of 28 mm laid the highest number of eggs with 1346 eggs over 14 batches. The number of eggs produced showed a significant positive relationship with animal length ($r_s = 0.96$, $P < 0.001$, $n = 7$) (Fig. 20A). However, there was no correlation between length and number of egg batches (Fig. 20B) and between number of batches and number of eggs produced. *I. zebra* is known to have large eggs and relatively low fecundity compared to other stylochids (Chintala & Kennedy 1993).

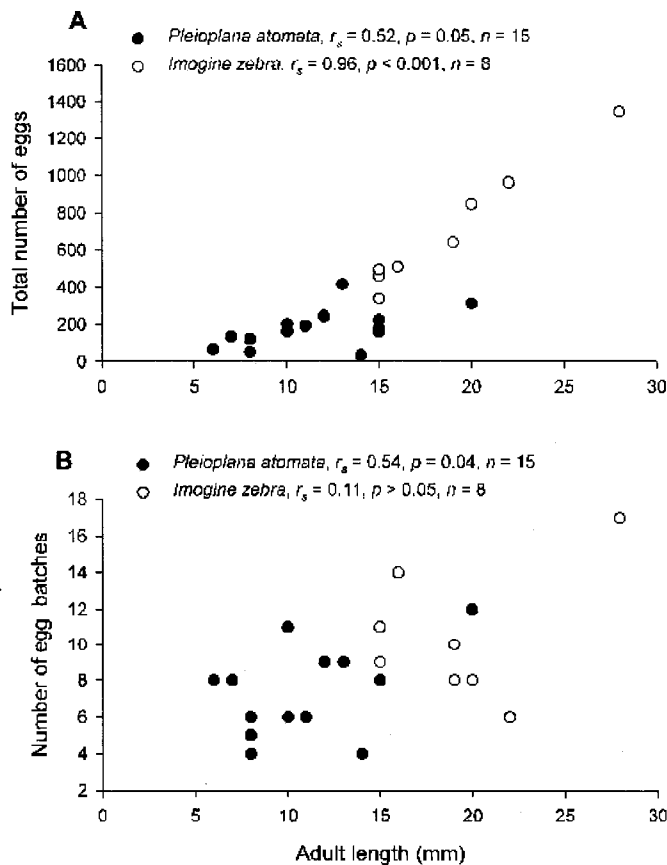


Figure 20. Relationship between adult length (mm) and (A) egg production, and (B) the number of egg batches in *Pleioplana atomata* and *Imogine zebra*.

Table 5. Comparison of modes of insemination and types of development with the presence or absence of a Lang's vesicle among various acotylean polyclads. Nomenclature *sensu* Faubel (1983); names in parenthesis correspond to names used in listed references.

Taxon	Lang's vesicle	Mode of insemination	Development	Reference
Family: Callioplanidae				
<i>Kaburakia excelsa</i> Bock, 1925	Absent	Unknown	Direct	Shinn (1987)
<i>Koinostylochus elongatus</i> (Kato, 1937) (= <i>Pseudostylochus elongatus</i> Kato, 1937)	Present; well-developed	Unknown	Direct	Kato (1940)
<i>Koinostylochus ostreophagus</i> (Hyman, 1955) (= <i>Pseudostylochus ostreophagus</i> Hyman, 1955)	Present; small	Unknown	Direct	Woelke (1956)
Family: Cryptocelidae				
<i>Cryptocelis alba</i> Lang, 1884	Absent	Unknown	Direct	Lang (1884)
Family: Discocelidae				
<i>Discocelis tigrina</i> (Blanchard, 1847)	Horseshoe-shaped	Unknown	Direct	Lang (1884)
Family: Euplanidae				
<i>Euplana gracilis</i> (Girard, 1850)	Absent	Unknown	Direct	Christensen (1971)
Family: Gnesioceridae				
<i>Echinoplana celerrima</i> Haswell, 1907	Small; rudimentary	Unknown	Direct	Ballarin & Galleni (1984)

Family: Leptoplanidae						
<i>Hoploplana villosa</i> (Lang, 1884)	Absent	Unknown	Direct			Kato (1940)
<i>Hoploplana inquilina</i> (Wheeler, 1894) (= <i>Planocera inquilina</i> Wheeler, 1894)	Absent	Dermal impregnation	Müller's larva			Wheeler (1894); Surface (1907)
<i>Leptoplana tremellaris</i> (Müller, 1774)	Small; rudimentary	Unknown	Direct			Selenka (1881)
Family: Notoplanidae						
<i>Notocomplana litoricola</i> (Heath and McGregor, 1912) (= <i>Freemania litoricola</i> (Heath and McGregor, 1912)	Present	Unknown	Direct			Shinn (1987)
<i>Notoplana alcinoi</i> (Schmidt, 1861)	Present	Unknown	Direct			Selenka (1881)
<i>Notoplana australis</i> (Schmarda, 1859)	Present	Unknown	Götte's larva			Anderson (1977)
<i>Notoplana delicata</i> (Yeri and Kaburaki, 1918)	Present	Unknown	Direct			Kato (1940)
<i>Notoplana humilis</i> (Stimpson, 1857)	Present	Copulation	Direct			Kato (1940)
Family: Planoceridae						
<i>Planocera multitentaculata</i> Kato, 1944	Highly reduced	Unknown	Müller's larva			Kato (1940)
<i>Planocera reticulata</i> (Stimpson, 1855)	Highly reduced	Unknown	Intra-capsular Müller's larva			Kato (1940)
Family: Pleioplanidae						
<i>Pleioplana atomata</i> (Müller OF, 1776) (= <i>Notoplana atomata</i> (Müller OF, 1776))	Present, well-	Copulation	Direct			Remane (1929); this study

	developed				
Family: Pseudostylochidae					
<i>Pseudostylochus intermedius</i> Kato, 1939	Present; well- developed	Unknown	Direct	Teshirogi et al. (1981)	
<i>Pseudostylochus obscurus</i> (Stimpson, 1857)	Present; well- developed	Copulation	Direct	Kato (1940)	
Family: Stylochidae					
<i>Distylochus pusillus</i> (Bock, 1913)	Absent	Unknown	Direct	Teshirogi et al. (1981)	
<i>Imogine aomori</i> Kato, 1937 (= <i>Stylochus aomori</i> Kato, 1937)	Absent	Unknown	Götte's larva	Kato (1940)	
<i>Imogine ijimai</i> Yeri and Kaburaki, 1918 (= <i>Stylochus ijimai</i> Yeri and Kaburaki, 1918)	Absent	Unknown	Götte's larva	Rho (1976)	
<i>Imogine lateotentare</i> Lee, Beal and Johnston, 2006	Absent	Unknown	Götte's larva	Lee et al. (2006)	
<i>Imogine mcgrathi</i> Jennings and Newman, 1996	Absent	Unknown	Götte's larva	Jennings & Newman (1996)	
<i>Imogine mediterraneus</i> Galleni, 1976 (= <i>Stylochus mediterraneus</i> Galleni, 1976)	Absent	Unknown	Götte's larva	Galleni (1976)	
<i>Imogine orientalis</i> Bock, 1913 (= <i>Stylochus orientalis</i> Bock, 1913)	Absent	Unknown	Direct	Chen et al. (1990)	
<i>Imogine uniporus</i> Kato, 1944	Absent	Copulation	Götte's larva	Kato (1940)	

(= <i>Stylochus uniporus</i> Kato, 1944)	Absent		Dermal impregnation	Direct	Lytwyn & McDermott (1976); this study
<i>Imogine zebra</i> (Verrill, 1882)	Absent		Unknown	Müller's larva	Provenzano (1959)
(= <i>Stylochus zebra</i> (Verrill, 1882))	Absent		Unknown	Götte's larva	Hofker (1930)
<i>Stylochus ellipticus</i> (Girard, 1850)	Absent		Copulation	Direct	Pearse & Wharton (1938)
<i>Stylochus flevensis</i> Hofker, 1930	Absent				
<i>Stylochus frontalis</i> Verrill, 1893 (= <i>S. inimicus</i> Palombi, 1931)	Absent		Unknown	Direct	Lang (1884)
<i>Stylochus neapolitanus</i> (Delle Chiaje, 1841)	Absent		Unknown	Götte's larva	Lang (1884)
<i>Stylochus piilidium</i> (Götte, 1881)	Absent		Unknown	Götte's larva	Merory & Newman (2005)
<i>Stylochus pygmaeus</i> Merory and Newman, 2005	Absent		Unknown	Götte's larva	Murina et al. (1995)
<i>Stylochus tauricus</i> Jacubowa, 1909	Absent				
Family: Stylochoplanidae					
<i>Stylochoplana maculata</i> (Quatrefage, 1845)	Present; round		Copulation	Direct	Remane (1929)
<i>Stylochoplana parasitica</i> Kato, 1935	Present; round		Unknown	Direct	Kato (1940)
Family: Theamidae					
<i>Theama mediterranea</i> Curini-Galletti et al. 2008	Absent		Unknown	Direct	Curini-Galletti et al. (2008)

Adult size is a positive predictor of female fecundity, with larger individuals producing more eggs. In *Pleioplana atomata*, the number of batches was also an indicator of reproductive success, but this was not the case for *Imogine zebra*. Another polyclad, *Stylochus ellipticus* (Girard, 1850) shows no size related female fecundity (Chintala & Kennedy 1993). However, the freshwater triclad *Dugesia gonocephala* (Duges, 1830) shows significantly increased reproductive success with increased size and actively selects mates based on size (Vreys & Michiels 1995). In hermaphrodites where copulations are reciprocal and size is a positive indicator of female fecundity, larger partners are favored. Thus, it is possible that size assortative precopulatory behavior is occurring in polyclads, too.

Developmental Time Line and Description of Embryonic Stages (Figs. 21, 22; Table 6)

Time from oviposition to hatching for *Pleioplana atomata* and *Imogine zebra* were 6 and 3 weeks, respectively (Table 6). Both are temperate species but the distribution of *P. atomata* in the Western Atlantic extends to higher latitudes (north of Cape Cod to Newfoundland), whereas *I. zebra* is found south of Cape Cod (Pollock 1998). Consequently, we reared the embryos of *P. atomata* at a temperature lower (15°C) than that of *I. zebra* embryos (22°C). Developmental times of many invertebrates have been shown to strongly correlate with temperature, with lower temperatures generally resulting in slowed development (Hoegh-Guldberg & Pearse 1995). In fact, preliminary observations reveal rapid developmental time lines for tropical and subtropical polyclads (Bolaños, pers. observation). Therefore, it is highly likely that the extended developmental times observed for *P. atomata* are a result of rearing temperatures. In the following, we discuss the developmental stages of *I. zebra* in detail. These observations were made on eggs reared at 22°C in the absence of adult animals. *P. atomata* underwent comparable stages, although at a delayed pace (Table 6).

Table 6. Time line of embryonic development of *Imogine zebra* and *Pleioplana atomata* (rearing temperature 22°C and 15°C, respectively).

Day	Hour	<i>Imogine zebra</i>	<i>Pleioplana atomata</i>
01	0	Oviposition	Oviposition
	3	Pseudopodia, blebbing	
	12	2 cells	
	16	4 cells	
	19.5	8 cells	
	22	16 cells	
02	13	32 cells	
02	22	64 cells	
03	0		2 cells
03	14		4 cells
04	0		8 cells
05		Gastrulation	
06		Cilia appear, slow rotation	
10		2 eyes	
11		Muscular contractions	Gastrulation
14		4 eyes, formation of mouth	Rotation by ciliary action
18		Formation of pharynx and intestinal branches	
21			Muscular contractions
22		Elongate, worm shape, hatching	
29			2 eyes
30			Contraction and Rotation
32			Strong muscular contractions, bending of worm
37			4 pairs of eyes
39			5 pairs of eyes
42			6 pairs of eyes - hatching starts

After oviposition and prior to the formation of polar bodies, numerous short pseudopodia appeared on the surface of the egg (*i. e.*, blebbing; Fig. 21A). These are extensions of the egg membrane and indicate the completion of meiosis of the egg cell. Egg blebbing has been described previously for many other polyclad species (Wheeler 1894; Kato 1940; Younossi-Hartenstein & Hartenstein 2000). The pseudopodia disappear once polar bodies have been formed and the zygote assumes a smooth, round shape (Kato 1940). First cleavage occurred 2 hours after oviposition and resulted in two equal-sized blastomeres (Fig. 21B). Cleavage progressed in a typical quartet spiral pattern (Fig. 21B-D); divisions were complete but unequal after the first two, as described for numerous other polyclad species (Surface 1907; Kato 1940; Anderson 1977; Boyer 1986). A solid morula formed on day 3 (Fig. 21E). Gastrulation via epiboly occurred on day 5 and the blastopore was clearly visible (Fig. 21F). On day 6, the developing embryos started to rotate slowly within the eggshells (88 s/complete turn), presumably due to ciliary action. At that time, the ectoderm was distinct, as was a concentration of large yolk globules in the interior of the embryo. During the next four days, the embryos began to rotate rapidly and on day 10, the first pair of eyes appeared (Fig. 21G). Also the color of the yolk gradually changed to brown, and eventually the granules were being absorbed rapidly. At this stage, the embryos occupied the entire space within the eggshells; strong muscular contractions became evident on day 11.

On day 14, a second pair of eyes appeared and a large, disc-shaped mouth was fully defined. Over the next 4 days, the embryo started to elongate, the pharynx and intestinal branches became noticeable, (Fig. 21H), a third pair of eyes developed, and the entire embryo was covered by cilia (Fig. 21I). By day 22, the embryos had taken on a characteristic, elongate worm-like shape with well-defined anterior and posterior ends. At 3 weeks, the juvenile *Imogine zebra* hatched (Fig. 21J). In *Pleiolana atomata* hatching occurred at 6 weeks. A 3-week time frame from oviposition to hatching is consistent with

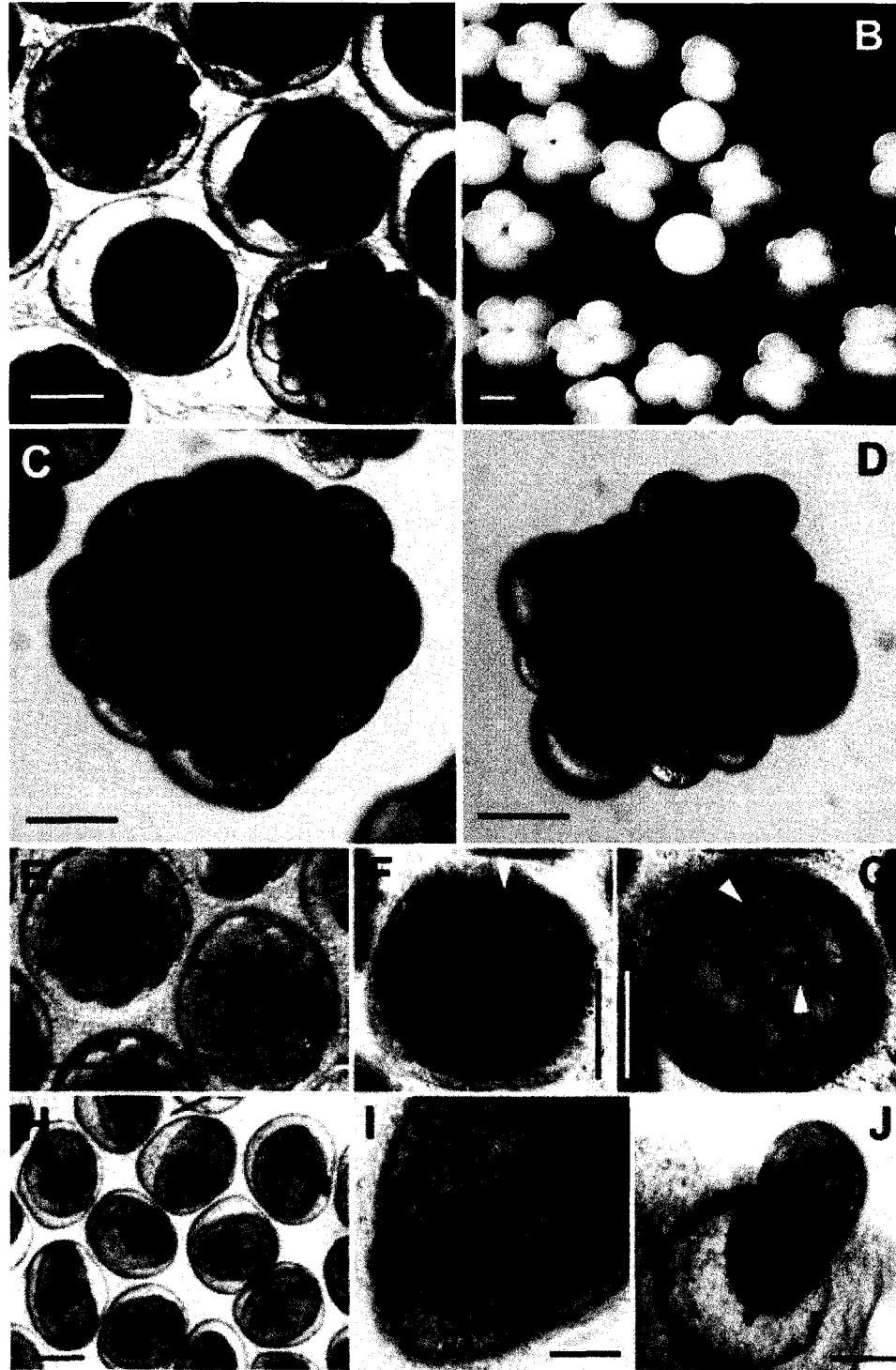


Figure 21. *Imogine zebra*, developmental stages. (A) egg blebbing, (B) 2-8 cell stage, (C) 16-cell stage, (D) 32-cell stage, (E) solid morulae, (F) gastrulation by epiboly, arrowhead = blastopore, (G) embryo with one pair of eyes (arrowheads), (H) embryos with noticeable pharynges, (I) embryo with 2 pairs of eyes, (J) juvenile worm hatching out of eggshell. Scale bars (C, D, I) 50 μm, all others 100 μm.

reports for other direct-developing polyclads. Kato (1940) noted the same time span for *Notoplana delicata* (Yeri & Kaburaki, 1918) and *N. humilis* (Stimpson, 1857), as well as for *Pseudostylochus obscurus* (Stimpson, 1857) and *Koinostylochus elongatus* (Kato, 1937) (reported under the name *Pseudostylochus elongatus*). Indirect-developing acotyleans however, tend to have shorter developmental times, e. g., 7-8 days for *Imogine aomori* Kato, 1937, *I. uniporus* Kato, 1944 (Kato, 1940), and *I. mcgrathi* Jennings and Newman, 1996 (Younossi-Hartenstein & Hartenstein, 2000).

Newly-hatched juveniles of *P. atomata* were about 490 μm long, tapered at both ends and dorso-ventrally flattened (Fig. 22A). Six pairs of eyes were arranged around the cerebral ganglion and anterior end of the pharynx. Sensory cilia were evenly positioned around the peripheral margin. *Imogine zebra* hatchlings were smaller (300 μm) in comparison, elongate and cylindrical (Fig. 22B). Three pairs of eyes were arranged anteriorly, cilia covered the external surface, and two longer caudal cilia were present at the posterior end (Fig. 22B).

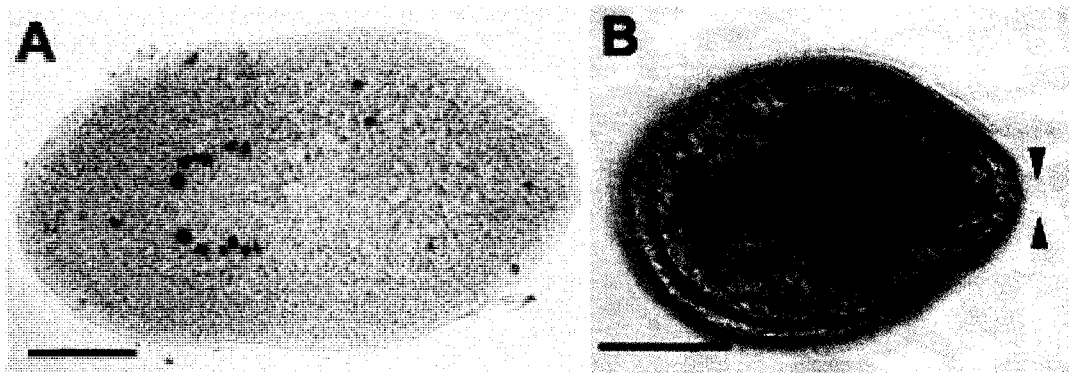


Figure 22. Hatchlings (A) *Pleioplana atomata*, and (B) *Imogine zebra*, arrowheads indicate caudal cilia. Scale bars, 100 μm .

The Polycladida is the only order among free-living flatworms to encompass species that develop indirectly through larvae as well as species that develop directly (Table 5). Larvae either have eight (Müller's larvae) or four (Götte's larvae) ciliated

lobes, which they use for swimming (Hyman 1951). Generally, Müller's larvae are found among members of the suborder Cotylea (polyclads with a ventral sucker present; Lang 1884), whereas Acotylea (ventral sucker absent; Lang 1884) may or may not pass through a larval stage. There is no phylogenetic correlation with family or even genus for the presence or absence of a larva among the Acotylea (Kato 1940; Ballarin & Galleni 1984; Table 5).

Notoplanidae has long been recognized as a heterogeneous assemblage of acotylean species, and taxonomic revisions of the family had been attempted by Bock (1913) and by Marcus & Marcus (1968). Based on the characteristically subdivided lining of the prostatic vesicle of *Notoplana atomata* (Müller OF, 1776), Faubel (1983) established the family Pleioplanidae and designated *N. atomata* as its type (*Pleioplana atomata*). Despite the re-classifications by Faubel (1983), the Notoplanidae still remains in need of revision. Therefore, we include comparisons on the development of genera closely related to *Pleioplana*. Development has been described for *Notoplana alcinoi* (Schmidt 1861), *N. delicata* (re-classified as *Pleioplana delicata* by Faubel, 1983), and *N. humilis* (re-classified as *Notocomplana humilis* by Faubel, 1983). All three species hatch as juvenile worms (Selenka 1881; Kato 1940). However, one species, *N. australis* (Schmarda, 1859) develops into a Götte's larva (Anderson 1977).

Similarly, the development of 16 species belonging to the family Stylochidae has been described (Table 5). Five of which are direct developers (Lang 1884; Pearse & Wharton 1938; Lytwyn & McDermott 1976; Teshirogi et al. 1981; Chen et al. 1990), the remaining species develop via a Götte's larva (Lang 1884; Hofker 1930; Kato 1940; Galleni 1976; Rho 1976; Murina et al. 1995; Jennings & Newman 1996; Merory & Newman 2005; Lee et al. 2006) and *Stylochus ellipticus* develops via a Müller's larva (Provenzano 1959). Among Leptoplanidae, a Müller's larva is known for *Hoploplana inquilina* (Wheeler, 1894) (Surface 1907) but direct development has been described for

its conspecific *H. villosa* (Lang, 1884) (Kato 1940). Thus, among acotyleans, mode of development is not taxonomically significant (Table 5). On the other hand, all cotylean species studied to date possess a Müller's larva; no Götte's larvae have been recorded for the group (Ruppert 1978).

Effects of Parental Care on Hatching Success

Parental care in the form of adult worms covering egg masses with their bodies (Fig. 23A) was observed for both species. In one instance, we observed guarding behavior of an *Imogine zebra* parent animal against a gastropod approaching the egg masses of the flatworm (Fig. 23B). For *Pleiolana atomata*, analysis of variance of Treatment 1 revealed a highly significant difference in hatching success between embryos with and without parental care (one-way ANOVA: $d.f._{1,54}$, $F = 19.44$, $P < 0.001$). The mean hatching success for embryos with parental care was 65.1% and without parental care 35.6% (Fig. 24A). The variation in hatching rate between eggs in the first three and second three batches of each individual was not significant (one-way ANOVA: $d.f._{1,70}$, $F = 1.82$, $P > 0.05$), therefore variation in viability between early- and late-laid eggs should not confound the results in Treatment 2. Taking variation of individual fecundity into account (Treatment 2), the two-way nested ANOVA showed significant care and individual effects; however, parental care was the most important factor (Fig. 24B, Table 7).

Imogine zebra adults covered the egg plates continually for up to 15 minutes after oviposition (Fig. 23A). Hatching success was not influenced by the presence or absence of the parent (Treatment 1, one-way ANOVA: $d.f._{1,34}$, $F = 0.01$, $P > 0.05$, Fig. 24C; Treatment 2, Fig. 24D and Table 7). However, the viability in the eggs of later-laid batches was significantly less than those laid earlier in the experiment (one-way

ANOVA: $d.f._{1,46}$, $F=6.09$, $P < 0.02$). As the effect of laying-sequence on egg viability is variable between species and can be significant, future studies should minimize this effect by using every other batch laid by the individual for the two conditions tested.

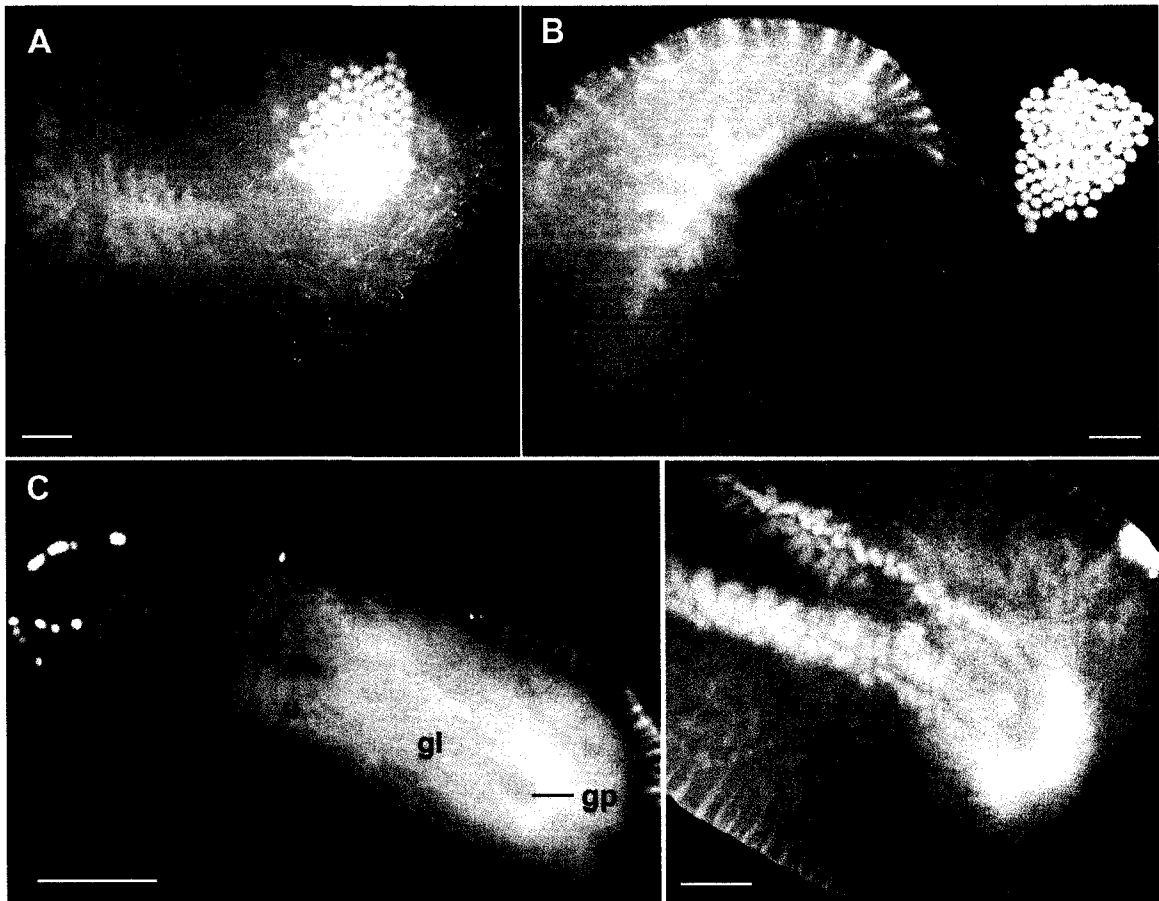


Figure 23. *Imogine zebra* parental care: (A) covering newly laid eggs, (B) attacking a gastropod near a recently laid egg plate, Scale bars, 1 mm. *Imogine zebra*, ventral views: (C) prior to oviposition, showing egg-filled uteri (u), filled ventral glands (gl), and gonopore (gp), scale bar 5 mm, (D) immediately after oviposition, showing marked decrease in gland contents, scale bar 2 mm.

One batch of eggs was removed from the adult immediately after oviposition and reared in filtered seawater. No eggshells formed and eggs were not arranged into a plate but cleavage proceeded normally. The function and the factors determining the evolution of covering behavior in polyclad parental care are not clear. Lee (2006) suggested it is a 'brooding' behavior affording protection to the developing embryos. However, it does not appear to be necessary for either development or hatching under laboratory conditions

(this study; Lee 2006). In the four species where the contribution of 'brooding' to hatching success has been quantified, only *Pleiolana atomata* showed a significantly increased hatching rate in the presence of a parent. In *Imogine zebra* (this study), *Echinoplana celerrima* and *Stylochus pygmaeus* Merory & Newman, 2005 (Lee 2006) hatching rates were not influenced by the presence or absence of parents. Because egg-covering behavior still occurred under controlled laboratory conditions where potential predators were absent, we infer that this behavior probably is not related to the protection of developing embryos.

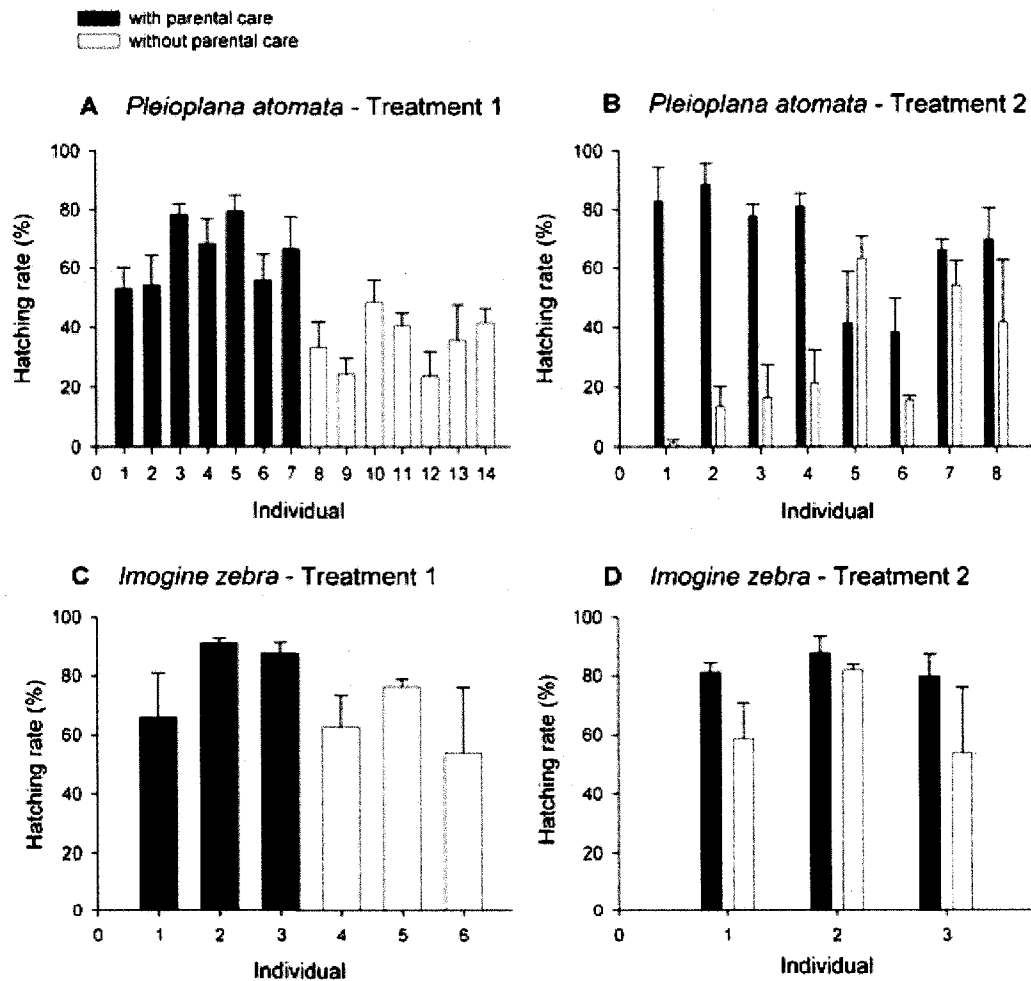


Figure 24. Variation in mean hatching rate ($\pm 1SE$) per batch of eggs with and without parental care in *Pleiolana atomata* and *Imogine zebra*. (A, C) Treatment 1 – egg hatching rate for all batches per individual; black bars – with parental care, white bars – without parental care, (B, D) Treatment 2 – three egg batches with parental care and three batches without parental care per individual.

In *Imogine zebra*, the role of covering behavior immediately after oviposition may be linked to eggshell formation and possibly the adhesion of egg masses to the substrate. Eggshell formation involves shell-forming granules in the egg, as well as secretions of shell glands discharged into the female atrium as eggs are deposited (Ishida & Teshirogi 1986). Soon after oviposition, the shell-forming granules swell through water absorption and separate from the egg to form the eggshell. Eggs of *I. zebra* collected within a minute of oviposition and developing in the absence of an adult, showed no eggshell formation. Still these eggs developed normally.

An examination of the ventral surface of *Imogine zebra* immediately prior to egg laying revealed not only uteri loaded with eggs but also glands prominently filled with materials ready for release (Fig. 23C). Immediately after oviposition, these glands showed a marked decrease in contents (Fig. 23D). Hence, it is possible that the covering behavior of the adult worm plays a role in the formation and tanning of eggshells. Additionally, covering behavior may result in the adhesion of the egg masses to the substrate. Kato (1940) too, describes adult worms covering cemented egg masses with gelatinous secretions from ventral glands. Further work on covering behavior and its role in shell formation clearly is required.

Table 7. Summary of two-way nested ANOVA of hatching rate of *Pleioplana atomata* and *Imogine zebra* eggs with and without parental care (Treatment 2).

	Source	d.f	MS	F	P
<i>Pleioplana atomata</i>	Parental care	1	0.016	14.59	0.002
	Animal (Parental care)	14	0.015	3.16	0.003
	Error	32	0.011		
	Total	47			
<i>Imogine zebra</i>	Parental care	1	0.0026	1.92	0.238
	Animal (Parental care)	4	0.0013	2.32	0.117
	Error	12	0.0006		
	Total	17			

CHAPTER III

TRUE POECILOLOGY IN A TROPICAL, FREE-LIVING FLATWORM (POLYCLADIDA: COTYLEA: *Pericelis cata*)¹

Introduction

Traditionally, developmental patterns of marine invertebrates are classified into direct and indirect development, with additional subdivisions based on nutrition (i.e., planktotrophic, lecithotrophic) and habitat (i.e., pelagic, benthic, brooded) (Chia 1974; Chia et al. 1996). Among free-living flatworms, Polycladida is the only order in which some species exhibit direct development with embryos hatching into benthic juveniles, whereas other species develop via a pelagic Müller's or Götte's larva. An eight-lobed Müller's larva is found among species of both suborders (Cotylea and Acotylea; with and without a ventral sucker, respectively; Lang 1884). In contrast, Götte's larvae displaying four lobes, are limited to acotyleans (Hyman 1951; Kato 1940; Ruppert 1978). Other than the intracapsular metamorphosis of the acotylean *Planocera reticulata*, in which embryos metamorphose through a larval stage while remaining inside the egg shell (Kato 1940), no other developmental variation has been described to date.

Poecilogony, defined as more than one developmental mode within one species, has been claimed for other marine invertebrates, especially mollusks and polychaetes (Hoagland & Robertson 1988; Chia et al. 1996). True poecilogony however, may actually

¹ Bolaños DM and Litvaitis MK. Embryonic Muscle Development in Direct and Indirect Developing Marine Flatworms (Platyhelminthes, Polycladida). Evolution and Development (*In review*)

be very rare, and many reported cases are in fact, due to cryptic species, sibling species, or systematic misidentifications (Hoagland & Robertson 1988; Chia et al. 1996). Currently, poecilogony has been established only for a few species of spionid polychaetes (Gibson et al. 1999; Schulze et al. 2000) and for sacoglossan gastropods (Jensen 1996; Krug 1998, 2007). These confirmed cases generally involve the production of short-lived, lecithotrophic larvae by one population, and planktotrophic larvae by a second population of the same species (Krug 2007). Here, we provide the first evidence for the simultaneous production of two different types of offspring (larva and metamorphosed larva/juvenile) by the polyclad flatworm, *Pericelis cata*.

Pericelis cata is a tropical cotylean polyclad, first reported from the coast of Curaçao (Marcus & Marcus 1968). It commonly is found in the Caribbean in rocky intertidal habitats and subtidally in coral rubble (Quiroga et al. 2004a, b; Rawlinson 2008). It is one of the largest polyclads and can measure up to 70mm in length and 50 mm in width when alive. In general, little is known about the biology and ecology of polyclads and *P. cata* is no exception. This species frequently is found in pairs, although single specimens are also common. *P. cata* prefers sedimentary rocks and coral rubble that are colonized by algae, tunicates and sponges. However, its interactions with other invertebrates or its feeding preferences remain to be identified.

A congeneric species, *Pericelis orbicularis*, is found sympatrically with *P. cata* in the wider Caribbean. The genus does contain two additional species (*P. byerleyana*, *P. hymanae*), however, they have been reported from the Pacific only. To assure that our embryological observations indeed are all based on *P. cata*, we include a short systematic re-description and histological comparison of *P. cata* and of *P. orbicularis*, as well as compare nucleotide sequences of the 28S rDNA gene of adults and hatchlings. The genetic matching of offspring to parent is even more important considering that *P. cata* most likely receives sperm by hypodermal impregnation, a phenomenon common

among cotylean flatworms (Hyman 1951; Michiels & Newman 1998). Partner animals stab each other with their penis papillae, usually on the dorsal surface and deposit spermatophores that are absorbed through the epidermis (Hyman 1951). Sperm travel through the parenchyma to fertilize the eggs. Hence, no copulatory lock-and-key mechanism assures the maintenance of species. Although polyclad flatworms are hermaphrodites, self-fertilization does not occur (Hyman 1951).

Materials and Methods

Details of specimen collection, maintenance in the laboratory and culture, and histology and taxonomic identifications have been described in Chapter I.

Adult polyclads were hand-collected from under rocks in the intertidal and shallow subtidal zones at Peanut Island (26° 46.428' N; 80° 82.608' W) and Sebastian Inlet (27° 51' 25.149 N; 80° 26' 45.0342 W) on the Atlantic coast of Florida, USA. Mature animals were placed individually into plastic bags, containing Millipore-filtered seawater, and the water was changed every other day. Masses of eggs were collected and embryonic development was followed. Eggs were observed and measured and developing embryos were followed through embryogenesis until hatching.

For taxonomic identifications, adult specimens were fixed on frozen 10% buffered formalin and preserved in 70 % ethanol. The segment containing the reproductive structures was dissected, embedded in paraffin, sagittally sectioned, and stained with hematoxylin and eosin. Sections were mounted and from where the reconstructions of the reproductive systems were derived. Taxonomic identifications were based on Marcus & Marcus (1968).

Molecular Tags

Small pieces of tissue excised from adult specimens and whole larvae were used for DNA analysis. High molecular weight DNA was extracted using Genomic Tips

(Qiagen Inc., Valencia, CA). The target nucleotide sequence was the D1-D2 expansion segment of the 28S rDNA gene (about 950 base pairs). Primer sequences (LSU D1, D2 fw1 and LSU D1, D2 rev2) can be found in Sonnenberg et al. (2007). DNA amplifications followed the protocol outlined in Litvaitis & Newman (2001). Amplicons were gel-purified and sent to a commercial lab for sequencing (Geneway Research LLC, Hayward, CA). Sequencing occurred in both directions. Trace files were edited using FinchTV (vers. 1.4; Geospiza Inc.) and sequences have been deposited in GenBank, under accession numbers EU679114 - EU679116.

Results

Development

Egg masses of *Pericelis cata* consisted of irregularly shaped discs (approx. 13 mm X 11 mm) of densely packed egg shells deposited as a single layer (Fig. 25A). The surface of the egg shells was smooth, decorated with brown speckles, and possessed a perfectly rounded lid-like operculum (Fig. 25B). As is common for polyclads, egg masses were covered and attached to the plastic bags by a thick gelatinous material of dark cream color. Zygotes were uniformly sized with a mean diameter of 105 μm ($n=120$) and varied from 3 to 5 eggs per egg shell (Fig. 25C). Time of development from oviposition to hatching was about 29 days.

Approximately 12 hours after oviposition, the eggs started to elongate and change their shapes, a phenomenon common during the time when eggs complete meiotic divisions and form the polar bodies (Fig. 25D). The first two cleavage divisions produced two and four equal blastomeres at about 20 and 34 hours, respectively (Figs. 25E, F). The third cleavage, at roughly 36 hours, was unequal, resulting in four micromeres on top of four macromeres (Fig. 25G).

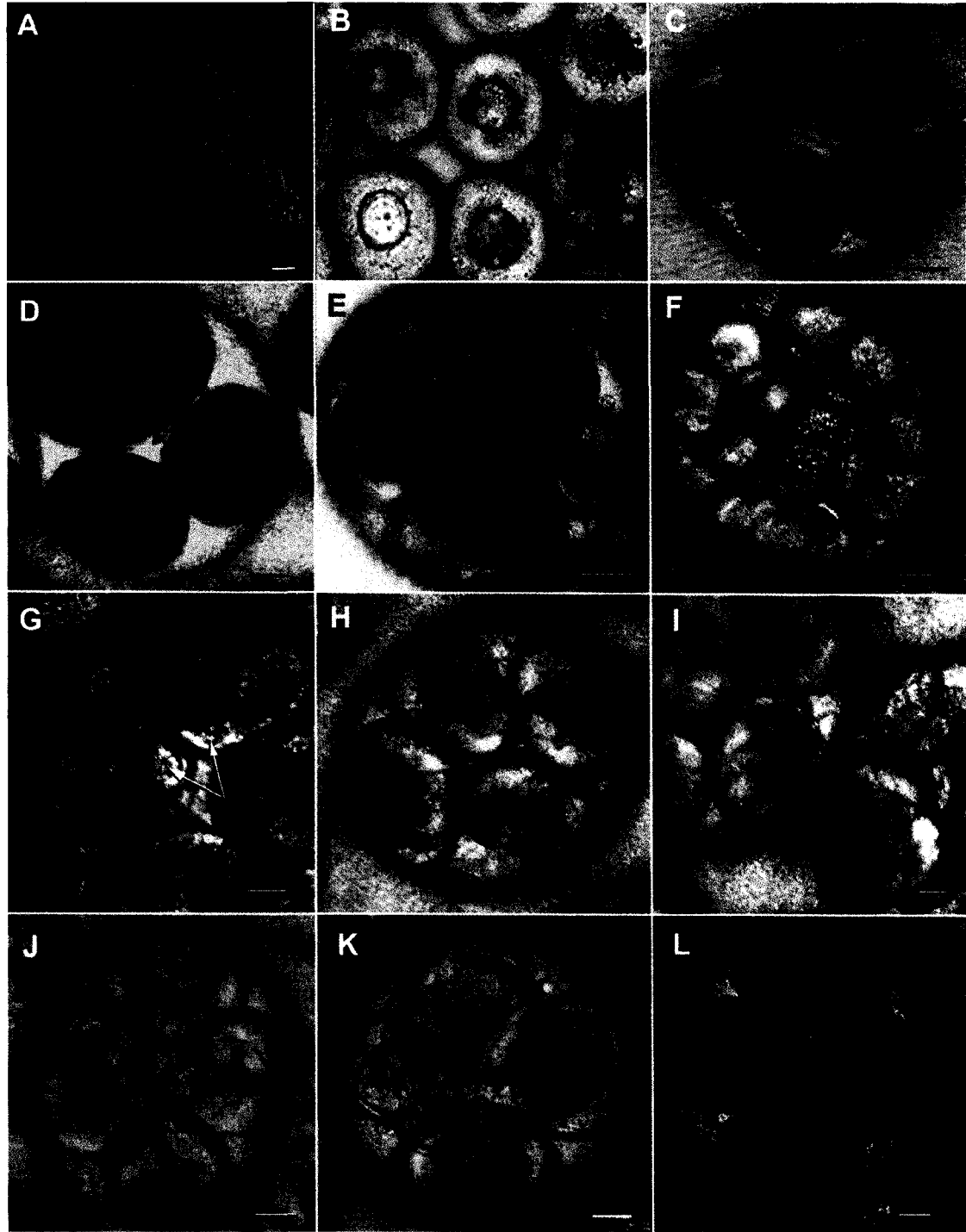


Figure 25. Early development of *Pericelis cata*. (A) Newly deposited, irregularly-shaped egg mass, scale bar, 1 cm; (B) empty egg shells with opened opercula, scale bar, 100 μ m; (C) multiple eggs within one egg shell, scale bar, 50 μ m; (D) elongation of eggs prior to first cleavage; (E) 2-cell stage; (F) 4-cell stage; (G) side view after third cleavage division, arrows indicate micromeres; (H) further flattening of eggs; (I) cell mass filling the embryos; (J) top view of the eggs filled with large cell masses; (K) eggs prior to epiboly; (L) early stereogastrula. Scale bars (D – L), 25 μ m.

Subsequent cleavages were not clearly distinguishable because the eggs flattened out, making it impossible to follow later spiral divisions (Fig. 25H). Eggs reached gastrulation at approximately 8 days. In other polyclads, gastrulation occurs by epiboly (Kato 1940; Hyman 1951), but in *Pericelis cata* it was impossible to identify the migration of micromeres towards the vegetal pole (Fig. 25K). At gastrulation, a large mass of cells filled the embryos (Figs. 25I,J). During the next three days, a stereogastrula was formed but only a few embryos were seen moving quite slowly, and no fast rotations were observed (Figs. 25L).

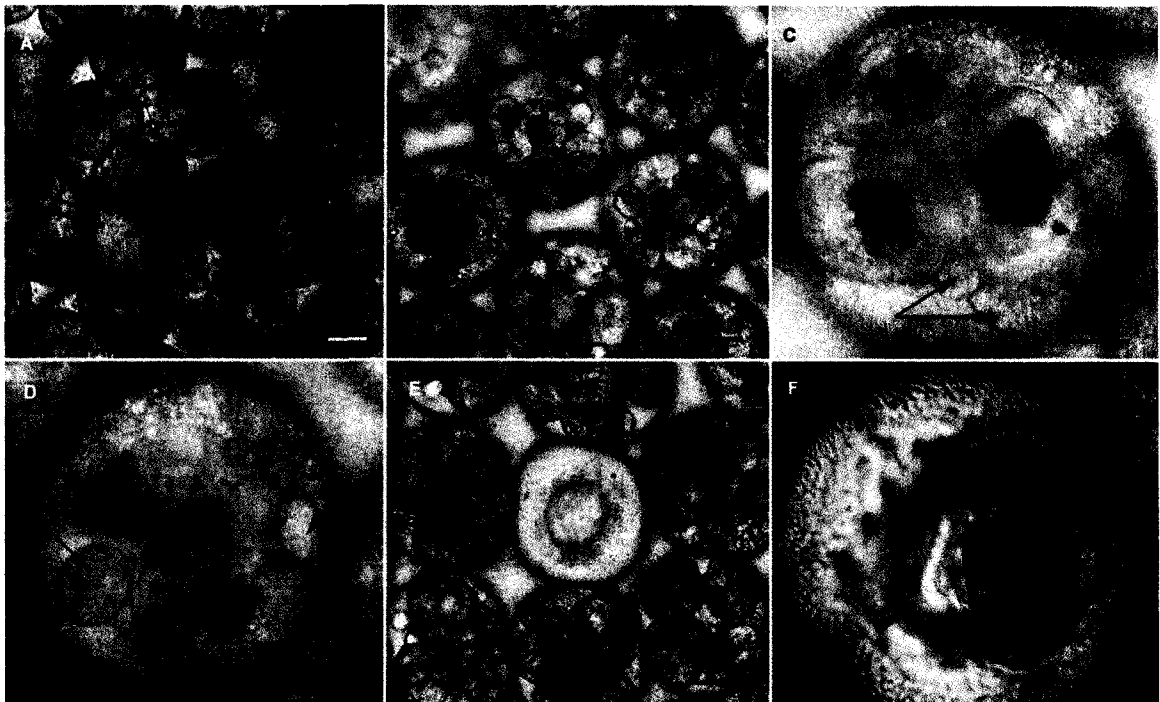


Figure 26. Embryonic development of *Pericelis cata*. (A) Late stereogastrula; (B) embryos after gastrulation with yolk droplets inside egg shells surrounding embryos, entire egg mass appears to consist of dead eggs; (C, D) embryos recognizable by dark pigmentation in body interior and by a single eye spot, arrows indicate yolk granules; (E) egg mass showing hatched eggs with open opercula and eggs still containing developing embryos, yolk granules still visible; (F) egg shell after hatching with one remaining larva, arrow heads indicate reduced lobes. Scale bars, 50 μ m.

Beginning at day 13, large extra-zygotic yolk droplets surrounded the embryos, diminishing the ability to detect any changes for the next 7-8 days; in fact, the embryos appeared to have died (Figs. 26A, B). At day 22, elongated embryos became

distinguishable by dark brown pigmentation in their body interiors, by prominent single eyespots, and by fast rotations (Figs. 26C, D). Yolk granules were still discernable and remained visible until hatching (Figs. 26D-F). Hatching of embryos started at day 26 and continued for 3 days. Egg shell opercula opened, releasing juvenile worms (87-95 μm)(Fig. 27A, B) and larvae (150-170 μm) (Fig. 28A-F) simultaneously from the same egg mass; however, it was not possible to determine if both were released from the same egg shell. Juveniles immediately settled to the bottom of the container, whereas larvae entered the water column. At no time did we observe only juveniles or only larvae hatching from an egg mass.

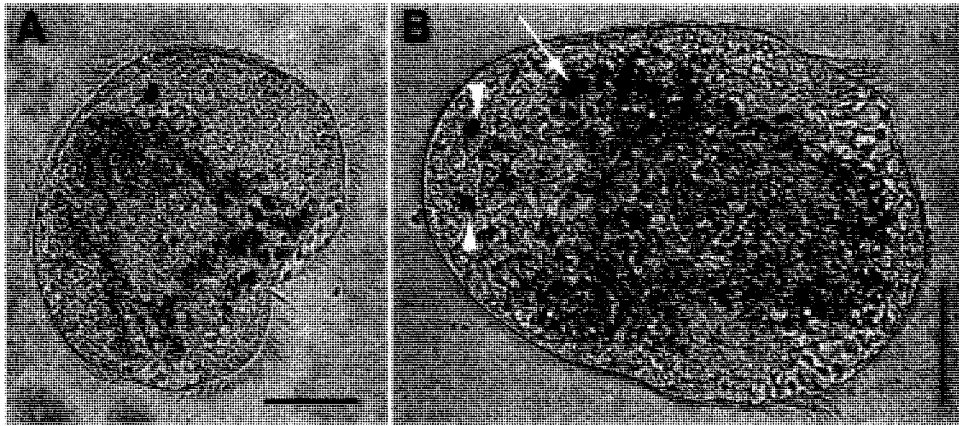


Figure 27. Postembryonic development of *Pericelis cata*. (A) Side view of juvenile with ciliated epidermis and single eye; (B) dorsal view of juvenile with single black eyespot (arrow), arrow heads indicating epidermal brown pigment which could be confused with eyes. Scale bars, 25 μm .

Larvae of *Pericelis cata* were morphologically different from the typical larvae of polyclads. They showed a single eyespot, rarely two. While still inside the egg shell, invaginations of the epidermis indicating the presence of lobes could be seen, however a typically shaped Müller's larva was not observed (Fig. 26F). Once hatched, the larvae could be viewed from different angles, and it was possible to identify a single eyespot, two small ventrolateral lobes (VLL) on each side of the circular mouth, a small mid-ventral lobe forming the oral hood (OH), and a pair of reduced lateral lobes (LL) (Figs. 28

A-D). There was no evidence of a mid-dorsal lobe. Numerous yolk granules were discernable inside the body, especially in the areas of the mouth and pharynx (Fig. 28D). Larvae possessed both apical and posterior sensory tufts, and a ciliated body with longer cilia around the lateral lobes (Fig. 28E, F). Juveniles, on the other hand, had long

cilia around the entire body but neither apical nor posterior tufts were observed. Juveniles hatched with one big noticeable eye and a relatively flattened body (Figs. 27A, B).

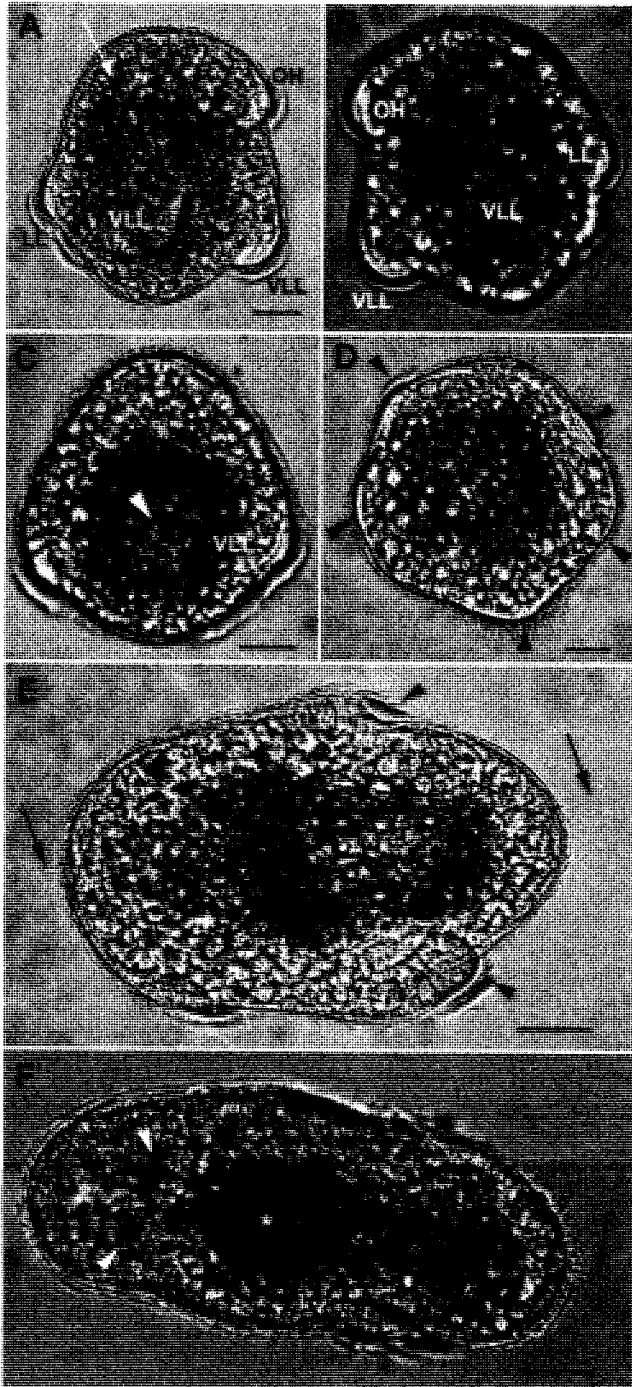


Figure 28. Postembryonic development of *Pericelis cata*. (A) Side view of newly-hatched larva with single eye spot (arrow), and lobes; (B) ventro-lateral view of the same larva; (C) ventral view, arrow head indicating mouth; (D) top view of larva, arrow heads indicating unpaired lobes, note yolk droplets in interior of the body; (E) larva with apical and posterior sensory tufts (arrows), and longer cilia on lateral lobes (arrow heads); (F) larva with elongated body, pigment present in narrow intestinal cavity (asterisk), and with two eyes (arrow heads) and posterior sensory tuft (arrow). (LL) lateral lobe; (OH) oral hood; (VLL) ventrolateral lobe. Scale bars, 25 μ m.

Species Identifications

Because *Pericelis cata* is found sympatrically with *P. orbicularis* we present a short review of their taxonomic separation based on adult external morphology, sagittal sections of mature reproductive systems, and on a 950-base pair segment of the 28S rDNA gene. We also extend the distributional range for the two species.

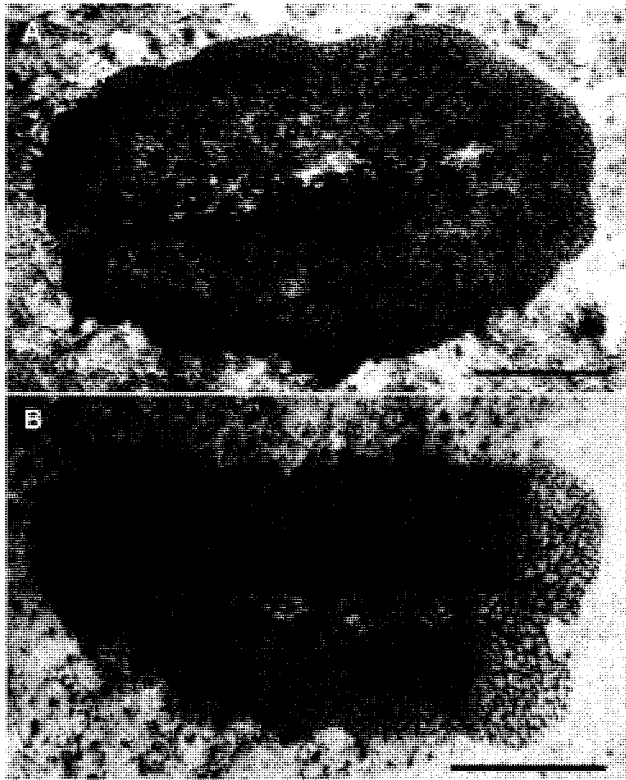


Figure 29. *In vivo* photographs of *Pericelis cata* (A) and *P. orbicularis* (B), showing color pattern and body shape of the animals. Note black pseudotentacles on *P. cata*. Scale bars, 1 cm.

Pericelis cata Marcus and Marcus, 1968

Material examined: Three mature specimens collected intertidally from under rocks at Peanut Island and Sebastian Inlet. Two specimens were sectioned sagittally (40mm x 23 mm; 30mm x 12mm), and one specimen was prepared as a whole mount (25mm x 10mm). Nine adult specimens collected throughout the range of the species were processed for DNA analysis, as were larvae from adults collected at Peanut Island and Sebastian Inlet, Florida.

Distribution: This species is widely distributed in the Caribbean and has been found in Curaçao, Panama, Jamaica, the US Virgin Islands, and Colombia. In this study, we extend its distribution to include the Atlantic coast of Florida.

Diagnosis: Oval body of brownish coloration, interrupted by light brown and white circles of irregular shape, a few black spots scattered over the dorsal surface (Fig. 29A). Conspicuous and well-separated pseudotentacles with black tips (Fig. 29A). Species characterized by the presence of a massive rounded seminal vesicle (840 µm) enclosed in a common muscular bulb with the prostatic vesicle. Prostatic vesicle small (100 x 166 µm), elongated, formed by the glandular epithelium and thick musculature surrounding the ejaculatory duct; curved, long and narrow male atrium with narrow gonopore; male pore very close to female pore (Fig. 30A). Shallow, narrow female atrium, short, slender vagina (100 µm in width) directed posteriorly and surrounded terminally by a weakly developed musculature; cement glands completely surround vagina and small defined cement pouch; uteri directed anteriorly; uterine vesicles present (Fig 30B). Sucker located posterior to female pore, 350 µm in width with few folds of the epithelium (Fig. 30C).

Pericelis orbicularis (Schmarda, 1859)

Material Examined: Three mature specimens collected intertidally from under the rocks at Reef Bay (18° 19.131' N; 64° 44.443' W), White Cliffs (18° 18.874' N; 64° 43.988' W), and Round Bay (18° 20.813' N; 64° 40.817' W) in the US Virgin Islands. One specimen was sectioned sagittally (40mm x 21mm) and the remaining part of the body was prepared as a whole mount. The other two specimens were prepared as whole mounts (37mm x 22mm; 40mm x 20mm). DNA was extracted from two adult specimens from St. John, US Virgin Islands.

Distribution: Reported from Jamaica, Port Aransas, Texas, and Key Biscayne, Florida.

We also found this species in St. John, US Virgin Islands.

Diagnosis: Oval body shape with ruffled margin of cream-colored background with dark brown pigment forming net-like pattern, no indication of pseudotentacles only small indentations at anterior margin (Fig. 29B). Large, oval seminal vesicle (792 x 500 μm) with enlarged ejaculatory duct; ejaculatory duct surrounded by a glandular epithelium but not by musculature. The prostatic vesicle is indistinguishable; curved, long and narrow male atrium with narrow gonopore; male pore very close to female pore (Fig. 30D). Shallow female atrium joined to a slender and well-developed vagina (190 μm in width) directed posteriorly and surrounded by well-developed musculature (Fig. 30E). Cement glands covering the entire female system extending to the male complex; bifurcated cement pouches (Fig. 30E). Uteri directed anteriorly; well developed uterine vesicle (up to 30 μm in diameter). Sucker located posterior to female pore, narrow and prominent, 170 μm in width (Fig. 30F).

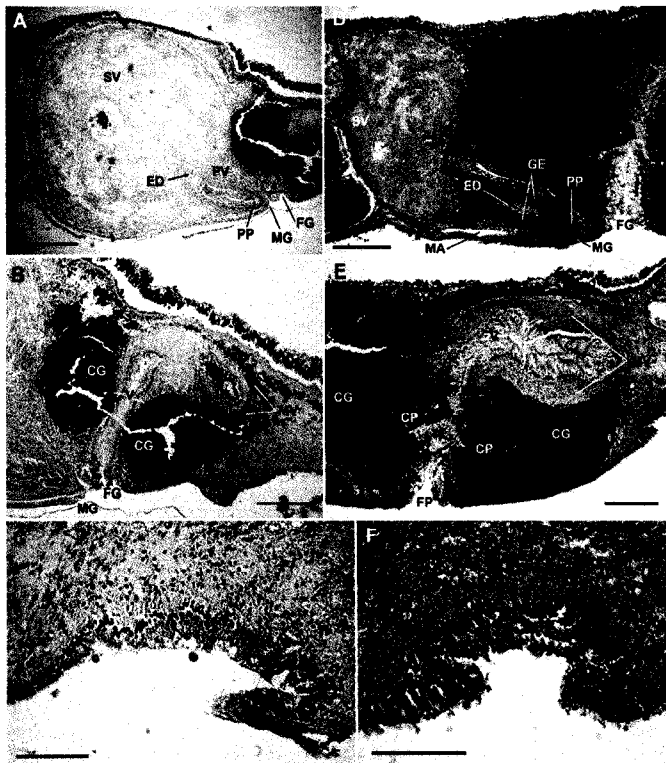


Figure 30. Morphological comparison between *Pericelis cata* (A-C) and *P. orbicularis* (D-F) based on characters of the male (A, D, scale bars 250 μm) and female (B, E, scale bars 100 μm) reproductive systems and the structure of the sucker (C, F, scale bars 100 μm); CG, cement glands; CP, cement pouch; ED, ejaculatory duct; FG, female gonopore; GE, glandular epithelium; MA, male atrium; MG, male gonopore, MV, musculature of vagina; PP, penis papilla, PV, prostatic vesicle; SV, seminal vesicle; V, vagina

A segment of about 950 base pairs of the 28 rDNA gene revealed 0% intra-specific variation for nine adult specimens of *Pericelis cata* and for the two *P. orbicularis* individuals. Larvae were clearly identified as belonging to *P. cata*. Inter-specific variation was 3.6%. In fact, we were able to identify 35 species-specific diagnostic nucleotide positions plus 3 insertion/deletion events. GenBank accession numbers are as follows: EU679114 (*Pericelis cata*, adult), EU679115 (*P. cata*, larva), and EU679116 (*P. orbicularis*, adult).

Discussion

The developmental dimorphism we describe for *Pericelis cata* represents a case of true poecilogony. Because larvae and juveniles hatched simultaneously throughout the three-day hatching period, we can conclude that development proceeds at different rates and that all embryos have to emerge once hatching is initiated regardless of their developmental state. It is likely that polyclad embryos can hatch at any viable stage (larva, juvenile) and that their hatching state is more dependent on physiological and possibly environmental conditions rather than on a fixed developmental program.

A possible physiological factor influencing hatching state may be food availability within the egg shell. Support for this hypothesis comes from Kato's (1940) study of *Planocera reticulata*, a polyclad with intracapsular metamorphosis. Clearly, the species retains a larval stage but hatches as a juvenile worm. Like *Pericelis cata*, *P. reticulata* also exhibits polyembryony; some of the embryos collapse and fragment into small yolk granules. Kato (1940) observed the living larvae feeding on the yolk granules, a behavior known as adelphophagy. We then would expect that in species with multiembryony where some embryos are used as nurse cells, hatching as a juvenile is the rule. However, this hypothesis needs further testing.

Regardless of the underlying reason, this case of dispersal polymorphism is of ecological interest, too. Chia et al. (1996) suppose that poecilogony must be quite common in marine invertebrates because intuitively, it may represent a bet-hedging strategy adaptive to fluctuating environmental conditions. New individuals are recruited to the parental habitat and are successful as long as conditions remain favorable. At the same time, siblings disperse as larvae to guarantee survival in case local conditions have changed since oviposition.

The most notable feature of the larvae of *Pericelis cata* was that almost all had only one eye, whereas three eyes characterize typical Müller's and Götte's larvae. Being a cotylean, *P. cata* should possess a Müller's larvae. However, a case could be made that the larva of *Pericelis cata* resembles the Götte's larva described for the acotylean *Stylochus tauricus*. According to Murina et al. (1995), larvae of *S. tauricus* possess four ciliated lobes and after a few days, a fifth lobe appears. Although the authors do not specifically provide details about the eyes of the larva, an examination of their drawings reveals a single eye, which persists without dividing to the juvenile stage even one month post-hatching. Additionally, Kato (1940) noted the formation of a single eyespot in the Götte's larvae of *S. uniporus* and *S. aomori*, commenting that this eyespot divides into two after metamorphosis. Unfortunately, juveniles of *P. cata* died soon after hatching and no division of the single eye was observed. Based on the foregoing, it is possible that the larvae of *P. cata* are of the Götte's-type and that the presence of one eye, even in juveniles, is due to a delayed division of the primary eye. The fact that a few larvae indeed showed a second eye is an indication that more eyes can form. To date, no Götte's larvae have been observed in cotyleans and *P. cata* would represent the first case.

At the same time, one has to consider that the distinctions between Götte's and Müller's larvae are not clear. For example, Lang (1884) proposes that Götte's larvae are

early stages of Müller's larvae and that they eventually will develop additional lobes. The claim has been discounted by Kato (1940) with his observations on *Stylochus uniporus* and *S. aomori*, two species that hatch as four-lobed Götte's larvae and metamorphose into juveniles without ever growing additional lobes. Considering several reports of other aberrant polyclad larvae (Kato 1940; Jägersten 1972; Galleni & Gremigni 1989; Murina et al. 1995), we prefer to consider the distinction between Götte's and Müller's larvae as artificial.

Polyclad flatworms are known to undergo spiral cleavage (Kato 1940; Hyman 1951, Chapters I & II); indeed they have been used as models to understand spiralian development (Boyer & Henry 1995; Henry et al. 1995). The zygote of *Pericelis cata* followed typical spiral cleavage during the first three divisions. However, subsequent divisions were difficult to observe because large amounts of extra-zygotic yolk appeared within the egg shells obscuring the developing embryo. Polyclad eggs are entolecithal i.e., all the yolk available to the developing embryo is deposited within the egg before oviposition (Lang 1884; Kato 1940; Hyman 1951). Hence, the appearance of extra-embryonic yolk appears unusual. It is possible that in *P. cata*, embryos passing through intracapsular metamorphosis require additional nutrition, normally not available in an entolecithal egg. We hypothesize that some eggs within each egg shell begin cleavage to produce macromeres and in this way concentrate the yolk that normally would be dispersed throughout the egg cytoplasm. At gastrulation, further development of these eggs is arrested and the large yolky macromeres provide supplemental nutrition to the remaining developing embryos. Such eggs are known as nurse eggs and have been described for mollusks (Chaparro & Paschke 1990 and references therein). Embryos that hatch as benthic juveniles use the yolk to complete their larval development. This is the case for many sacoglossan opisthobranchs in which extra yolk deposited into the egg capsule provides supplemental nutrition to the developing larvae (Clark & Goetzfried

1978; Clark & Jensen 1981; Boucher 1983). Although we could not positively confirm this assumption for *P. cata*, indirect evidence comes from the presence of yolk droplets in the areas of the pharynx and the mouth of hatched larvae and juveniles.

A second possibility for the origin of the extra-zygotic yolk may be via a process observed in *Macrostomum appendiculatum*, a member of the immediate sister group of polyclads. Like polyclads, *M. appendiculatum* also produces entolecithal eggs. Gastrulation occurs at the 16-cell stage via inverted epiboly, in which the 12 micromeres sink into four large, yolk-filled, vegetal macromeres (Thomas 1986; Galleni & Gremigni 1989). Subsequently, the macromeres flatten to envelop the micromeres completely, a process that resembles epiboly in neophoran flatworms (Thomas 1986). It is possible that a similar process occurs during gastrulation in *Pericelis cata* producing the observed extra amounts of yolk. In *M. appendiculatum* though, the extra yolk usually disappears because it is incorporated during the formation of the brain, pharynx, genital apparatus, and epidermis (Thomas 1986). In *P. cata* the extra-zygotic yolk persisted throughout development and juveniles and larvae were seen immersed among large granules until hatching. Additionally, as stated above, the fact that small yolk granules were seen inside the larvae and juveniles in the areas of the pharynx and mouth suggests that this material is used as a nutritional source. Hence, we favor the hypothesis of nurse eggs as the source of extra-zygotic yolk.

Pericelidae Laidlaw, 1902 is a monogeneric family currently established into the suborder Cotylea because of the presence of a sucker, pseudotentacles, and numerous uterine vesicles. However, some features of its member species are also representative of species in the suborder Acotylea. In fact, a recent phylogeny of the cotyleans based on morphological characters suggested that the sister group of all cotyleans is a clade including *Pericelis*, *Marcusia*, and *Anonymus* (Rawlinson & Litvaitis 2008). Acotylean-like characters of Pericelidae include a ruffled pharynx that is located centrally (ruffled

pharynges in other cotyleans generally are located well anteriorly), uteri that are directed anteriorly (in general, cotylean uteri are directed posteriorly), and the presence of marginal eyes around the entire body.

Because two of the four species of *Pericelis* co-occur in the Caribbean, we have provided a short re-description of *Pericelis cata* and *P. orbicularis* and supplied molecular tags that clearly distinguish the two species. Externally, the two species are separated by color pattern and by the shape and coloration of the pseudotentacles. Internally, *P. cata* differs from *P. orbicularis* because of a massive rounded seminal vesicle, and because of the considerable thick musculature that encloses the ejaculatory duct, allowing the duct to act as an elongated prostatic vesicle. In *P. orbicularis* on the other hand, the seminal vesicle is elongated and the musculature of the ejaculatory duct is not developed. In fact, the seminal vesicle simply enlarges to form the ejaculatory duct, which is surrounded by a glandular epithelium and which connects directly with the penis papilla. The sucker is the main characteristic of cotyleans and compared with other members of the suborder, is poorly developed in *Pericelis*. In sagittal sections, a shallow depression posterior to the female gonopore is the only evidence of the sucker, which is hardly visible in live specimens. Finally, an alignment of about 950 base pairs of the 28S rDNA gene of *P. cata* and *P. orbicularis* revealed 35 species-specific changes. Although we maintain the systematic position of Pericelidae as a basal clade within Cotylea, the family is clearly in need of taxonomic review.

CHAPTER IV

EMBRYONIC MUSCLE DEVELOPMENT IN DIRECT AND INDIRECT DEVELOPING MARINE FLATWORMS (PLATYHELMINTHES, POLYCLADIDA)¹

Introduction

Rhabditophora (Platyhelminthes other than Acoelomorpha and Catenulida; Ehlers 1986) comprise a diverse group of flatworms characterized by highly variable body shapes that are mostly due to the body wall musculature. Functionally, their body wall musculature is not only linked to body shape but also to locomotion, feeding, copulation and egg-laying (Hyman 1951). Fluorescent dye-conjugated phalloidin has been used to study the muscle organization of free-living rhabditophorans, such as *Macrostomum hystricinum marinum* and *Hoploplana inquilina* (Rieger et al. 1994; Reiter et al. 1996), facultative parasites, such as *Urastoma cyprinae* (Hooge & Tyler 1999a) and obligate parasites, e. g., *Fasciola hepatica*, *Diplostomum pseudospathaceum* and *Schistosoma mansoni* (Czubaj & Niewiadomska 1997; Mair et al. 1998a, b; 2000, 2003).

Overall, the rhabditophoran body wall musculature is composed of layers of outer circular and inner longitudinal fibers organized into an orthogonal, grid-like pattern (Hyman 1951, Prudhoe 1985). This organization is considered the ground pattern for many flattened vermiforms (all lineages within Platyhelminthes, Acoelomorpha, Nemertea) (Hyman 1951; Rieger et al. 1991a; Rieger et al. 1994; Hooge 2001).

¹ Bolaños DM and Litvaitis MK. Embryonic Muscle Development in Direct and Indirect Developing Marine Flatworms (Platyhelminthes, Polycladida). Evolution and Development (*In review*)

Additionally, the rhabditophoran body wall may contain one or several sheets of diagonally oriented muscle fibers located between and below the circular and longitudinal layers (Hooge 2001). However, these diagonal layers may be reduced or may even be completely absent (for a comprehensive review of adult flatworm body wall musculature, see Hooge & Tyler 2003). The orientation of the diagonal fibers is such that they angle clockwise and counterclockwise around the animal (Rieger et al. 1991a), crossing over each other and forming a second grid, offset by about 55° from the orthogonal pattern. Finally, a parenchymal musculature formed by muscle fibers traversing the central parenchyma and consisting of well-developed dorsoventral, transverse and ventral longitudinal muscles is present (Hyman 1951).

Although descriptions of flatworm muscle organization have received considerable attention, studies of pattern formation during developmental myogenesis remain scarce (Reiter et al. 1996; Younossi-Hartenstein & Hartenstein 2000; Hartenstein & Jones 2003), and are mostly focused on the emerging model species, *Macrostomum hystricinum marinum* (Rieger et al. 1991a, 1994; Reiter et al. 1996; Morris et al. 2004). In a comparative study on the differentiation of body wall musculature in *M. h. marinum* and *Hoploplana inquilina*, Reiter et al. (1996) found that muscle development varied depending on if the worms hatched as miniature adults or as larvae. *Hoploplana inquilina*, is an acotylean polyclad with indirect development via a Müller's larva, the other species belongs to the direct-developing Macrostomida. Polycladida and Macrostomida are immediate sister taxa and represent early lineages within the Rhabditophora (Carranza et al. 1997; Litvaitis & Rohde 1999). Furthermore, they both belong to the Archoophora, a grouping based on an organizational grade derived from the homocellular arrangement of female gonads and the production of entolecithal eggs (Hyman 1951). This organizational grade contrasts with the Neophora, which are characterized by heterocellular gonads and ectolecithal eggs and include all remaining

rhabditophorans (Hyman 1951). Both terms however, carry no systematic value and are best used for developmental descriptions only.

In the larvae of the polyclad, *H. inquilina* muscle development was strongly dependent on two founder muscles bands that were laid down in a bilaterally symmetrical pattern along the longitudinal axis of the animal. Following this initial muscle guide, two circular rings of muscles developed: one anteriorly, demarcating the rostral end from the trunk region, the other more posteriorly at the junction between trunk and tail end (Reiter et al. 1996). This is in contrast to observations in the direct-developing *M. h. marinum*, where several longitudinal muscle bands could be seen to which many circular fibers attached at right angles (Reiter et al. 1996). In this direct-developing pattern, no initial bilateral symmetry or trunk demarcations are evident. It is tempting to speculate that such observed differences in muscle development are related to differences in developmental mode (direct vs. indirect). However, at present, there is no evidence to either support or refute such speculations.

In this study, we examined embryonic muscle differentiation in *Maritigrella crozieri* and *Melloplana ferruginea*, providing the first comparative analysis on pattern formation during polyclad myogenesis. *Maritigrella crozieri* is characterized by a Müller's larva and is grouped into the suborder Cotylea (polyclads with a ventral sucker, Lang 1884), whereas *M. ferruginea* is a direct-developing acotylean (polyclads without a ventral sucker, Lang 1884). The main purpose of this study was to provide new developmental and morphological data that contribute to the understanding of body wall muscle formation during embryogenesis, and to determine if observed differences in muscle development are related to differences in developmental mode. Additionally, we also compared our findings to muscle development in other flatworms and other Spiralia.

Materials and Methods

Collection

Adult worms of *Maritigrella crozieri* were collected from submerged hanging lines at Little Jim's Marina, Fort Pierce, Florida, USA (27° 28.42' N, 80° 18.4' W), where they were associated with their prey items, the ascidian *Ecteinascidia turbinata*. Adult specimens of *Melloplana ferruginea* were collected from under rocks in the intertidal and shallow subtidal zones at Peanut Island (26° 46.428' N; 80° 82.608' W) on the Atlantic coast of Florida, USA. Animals were picked up using a soft paintbrush to avoid autolysis and were placed individually into small plastic bags filled with seawater. Reproductive maturity of individuals was determined by inspecting the ventral sides of the worms for eggs. When present, eggs are easily visible in the oviducts. Egg-bearing specimens were acclimated for two days in individual plastic bags containing Millipore-filtered seawater.

Culture

After acclimatization, mature specimens were placed into Petri dishes and their uteri were punctured with small tungsten needles. This caused the release of zygotes without egg capsules (naked eggs). The eggs were transferred into small gelatin-coated Petri dishes of Millipore filtered seawater that contained 200 µg/ml streptomycin and 60 µg/ml penicillin to prevent microbial or fungal growth. Embryonic development was followed until the formation of larvae and juveniles. Eggs were observed under a Leica DMLB microscope equipped with a Nikon CoolPix 8700.

Phalloidin staining and confocal microscopy

Embryos, larvae and juveniles were fixed at differing developmental stages at room temperature in 4% paraformaldehyde in 0.01M phosphate buffer (PBS; pH 7.4) for

45 min. Specimens were rinsed (3X) for 15 min with 0.01M PBS, permeabilized for 1h in 0.2% Triton X-100 in PBS, stained for 2 h or overnight with Alexa Fluor 488 phalloidin (Molecular Probes, Eugene, OR, USA), and rinsed twice for 10 min with PBS. Specimens were mounted on glass slides in Gel/Mount (Biomedica Corp.), and digital image acquisition and analysis were performed using a Zeiss LSM 510 confocal microscope (UNH Instrumentation Center). Samples were excited with a 488 nm multi-line argon-ion laser and emitted fluorescence was collected using a 505-530 nm bandpass filter. Measurements of the specimens were taken with an ocular micrometer prior to fixation. For each species, four specimens were examined.

Results

Regardless of developmental mode, the final muscle orientation and arrangement of layers resulted in an orthogonal pattern in both species. However, the underlying processes of myogenesis differed considerably between the two. *Maritigrella crozieri* hatched as an eight-lobed Müller's larva seven days after eggs were deposited, whereas *Melloplana ferruginea* emerged as a juvenile worm about 22 days after oviposition. Myogenesis in *M. crozieri* resulted in a significantly complex musculature followed by major changes post-hatching. Juvenile worms of *M. ferruginea* on the other hand, emerged with a typical body wall musculature of circular, longitudinal, and diagonal muscle fibers, similar to that in the adult worm. We were able to identify different developmental stages in both species by the appearance of external morphological characters. To conform to published studies on embryonic myogenesis in archoophorans (Reiter et al. 1996; Ladurner & Rieger 2000), we define developmental stages as a percentage of total developmental time, where 100% represents the time from oviposition to hatching. However, we also include time since oviposition in h to allow for comparisons with studies of other spiralian (Maslakova et al. 2004; Bergter &

Paululat 2007). For ease of comparison, a summary of the major events discernable by phalloidin staining can be found in Table 1.

Muscle differentiation in *Maritigrella crozieri* (Table 1)

After oviposition (0% development), zygotes (n=50), which on average measure 134 μm in diameter, underwent holoblastic, spiral cleavage, eventually dividing the eggs into 64 cells. At about 17% of development (28h), the eggs contained small amounts of yolk, which emitted a dull autofluorescence. Gastrulation began at approximately 30% of development (50h). Egg surfaces became irregularly shaped, and a faintly-staining, polygonal pattern became visible (Fig. 31A). Actin filaments inserting into the zonulae adhaerentes were evident (Fig. 31B). Additional actin was distributed all over the egg surface but was present in higher concentrations in some regions. No muscle fibers were observed immediately after epiboly. However, shortly after gastrulation (approx. 36% development, 60h), a few small myoblasts were apparent that were randomly dispersed over the egg.

Once gastrulation was completed, the first cilia appeared and minimal movement of the embryos was observed (37-43% of development, 62-72h). A brightly-staining, primary circular muscle band was recognized (Fig. 31C), with unorganized muscle fibers distinguishable in the center of the developing egg. During this stage, many individual muscle fibers appeared and the epidermis was easily recognized by the polygonal outlines of epithelial cells. At about 43% of development (72h), secondary fibers derived from the primary circular band were detectable, although still no contractions were observed at this point (Fig. 31D). Additionally, unorganized weakly staining fibers could be distinguished deep within the embryo. Also during this stage, a primary longitudinal muscle formed which ran whip-like along the entire length of the embryo from the

anterior to the posterior end and looping back to the anterior pole (Fig. 31D).

Presumably, this looped fiber extended into the area of a future larval lobe.

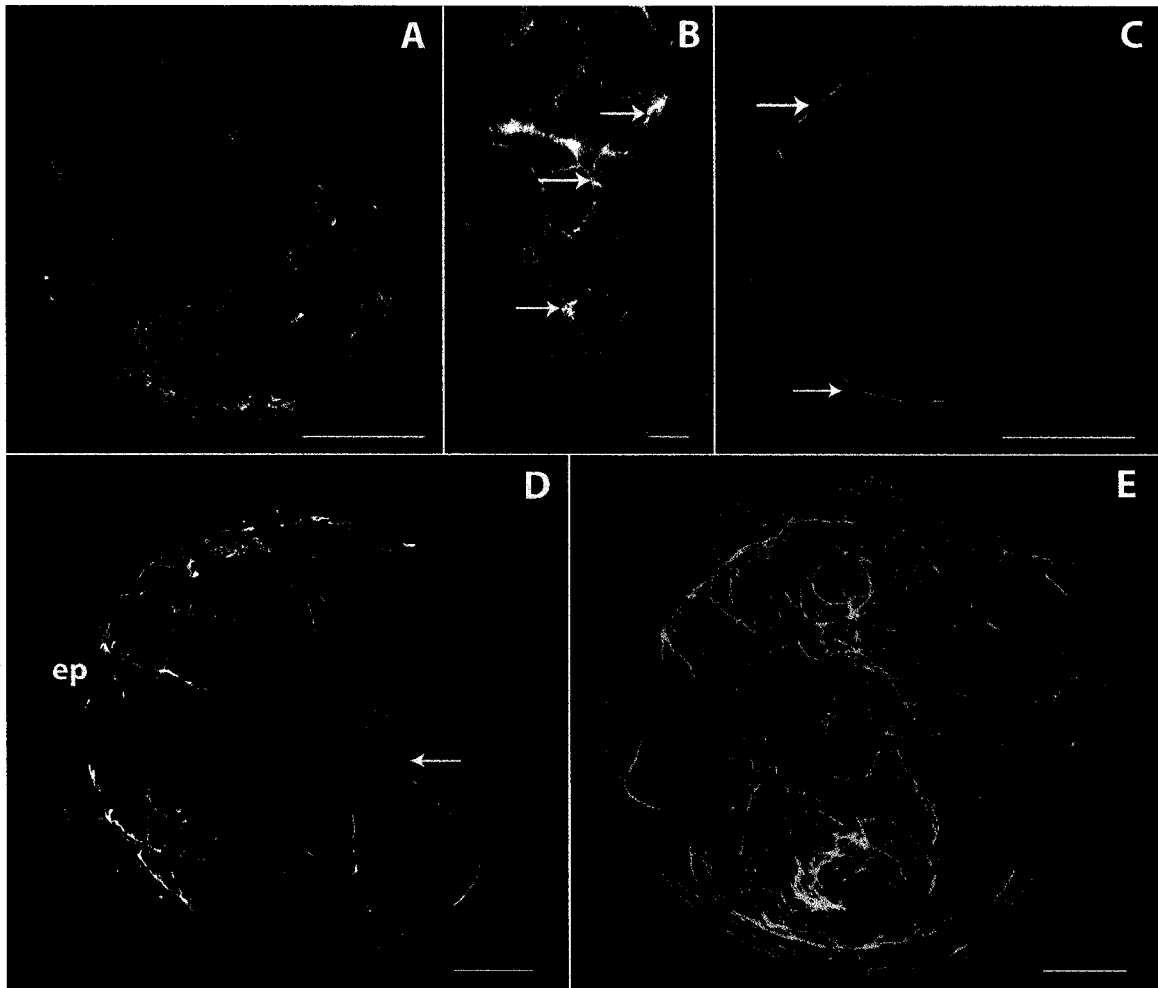


Figure 31. Early embryonic development of *Maritigrella crozieri*. (A) Egg surface showing polygonal outlines of epidermal cells. Scale bar, 50 μ m; (B) higher magnification of egg surface, arrows indicate actin in the zonulae adhaerentes. Scale bar, 10 μ m; (C) egg after gastrulation, arrows indicate primary circular muscle band. Scale bar, 50 μ m; (D) primary longitudinal muscle (arrow) extending whip-like along the entire length of the embryo; arrowheads indicate circular muscles (E) concentric organization of larval lobe musculature. ep: epidermis. Scale bars, 25 μ m.

During a transitional period at 44% of development (74h, day 4), the embryo still appeared spherical but muscle fibers began to form concentrically in the lateral larval lobes (Fig. 31E). Ciliary action caused the embryos to slowly rotate within the egg capsules. At this stage, a single eye was present and there was no indication of any musculature associated with the mouth.

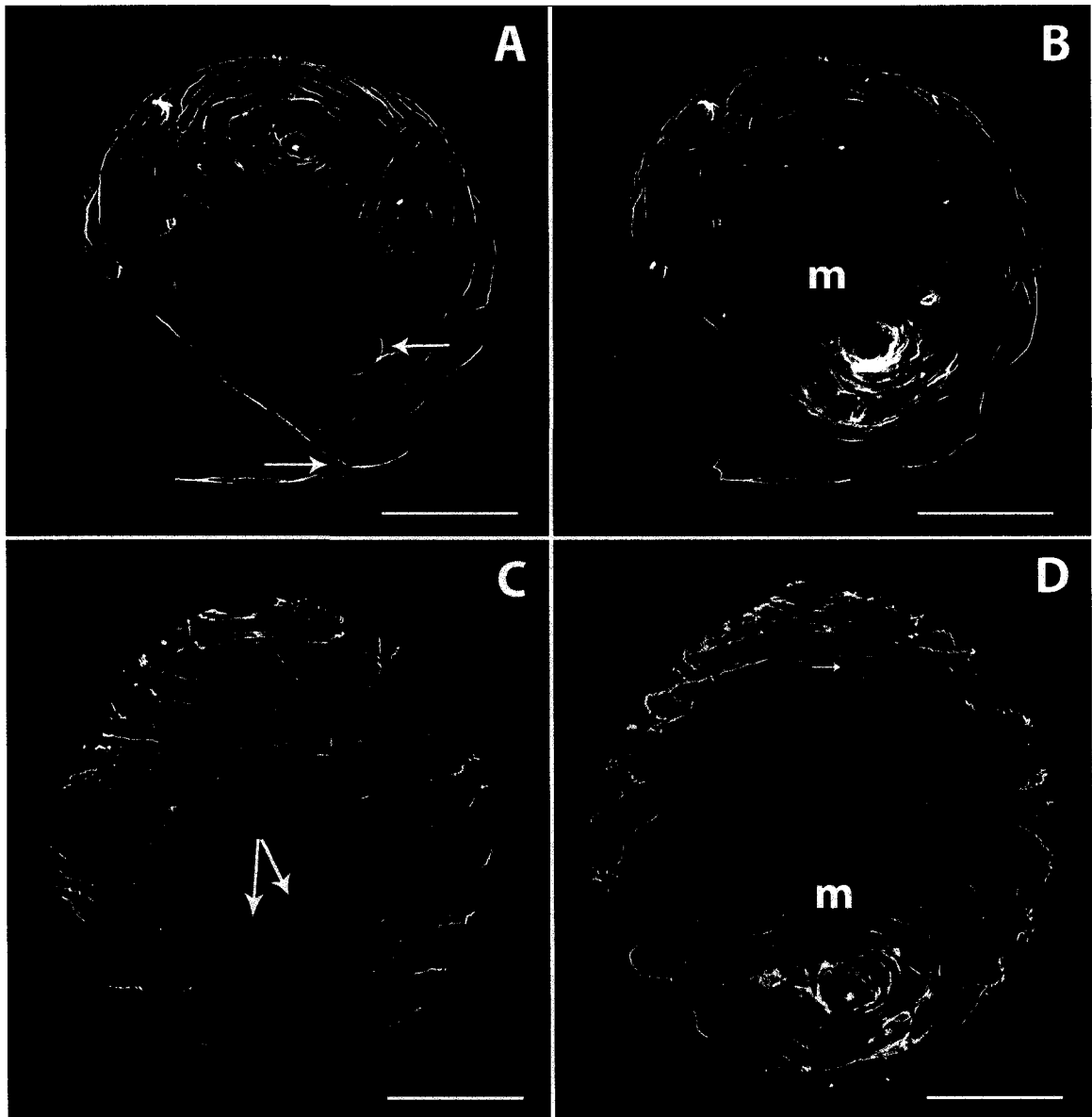


Figure 32. Early embryonic development of *Maritigrella crozieri*. (A) Muscle fibers of larval lobes, showing spiral organization. Arrows indicate bifurcation of the circular fibers; (B) ventral view showing dense musculature of the mouth; (C) dorsal view of circular muscle fiber distributed over the embryo. Arrows indicate small circular fibers extending medially and returning to the edges of the embryo; (D) ventral view showing organization of the circular fiber bands. Arrow pointing at a short anterior longitudinal fiber. Bright dots around the mouth represent insertion points of future longitudinal muscles, m: mouth. All scale bars, 50 μ m.

Although at 45-52% of development (75-88h, day 4), larval lobes were not yet completely formed, their musculature composed of circular muscles fibers running in a simple spiral, was evident (Fig. 32A). During that time, additional longitudinal muscles

began their development; they could be distinguished as faintly staining branches. Thicker, circular fibers encircled the periphery of the embryo, bifurcating and forming additional muscle fibers (Figs. 32A). At this stage, first contractions of the body were evident, faster rotations of the embryos were observed, and a second eye could be distinguished. On the ventral side of the embryo, muscle fibers associated with the mouth were discernable (Fig. 32B). The muscles of the mouth were seen as a separate muscular field, in which concentrically distributed muscle fibers were more strongly developed than the circular muscle fibers of the lobes. Unlike the circular muscle fibers of the larval lobes, oral muscle fibers did not run in a spiral fashion but instead were densely packed, close to each other, and formed a sphincter surrounding an opening that extended deep into the embryo (Fig. 32B).

At 53-60% of development (89-101h, day 4), the shape of the embryo was slightly ovoid, and the arrangement of the circular muscle system became clearly evident (Figs. 32C, D). Apically, these circular rings of muscles were more abundant and consisted of 3 to 5 individual muscle fibers. At this stage, a few muscle bands completely surrounded the embryo but towards the posterior end they were less abundant and still incomplete (Figs. 32C, D). In the area of the equator, one circular fiber extended from each side of the embryo, towards the center of the body, turning there, and returning to the margin. These fibers are probably associated with the lobes (Fig. 32C). At the very posterior end, three brighter areas were seen associated with the oral musculature. It is likely that these areas will give rise to the dilator muscles of the mouth (Fig. 32D). Furthermore, small, bright dots were localized around the muscles of the mouth, which represented the insertion points of future longitudinal muscles. Some of the circular muscle bands bifurcated and gave rise to new small fibers. At the apical end, one short onspicuous fiber extended longitudinally (Figs. 32C, D). In a maximum projection of the

embryo, the entire circular muscle system was visualized, showing that fibers were densely packed and not evenly spaced (Fig. 32D).

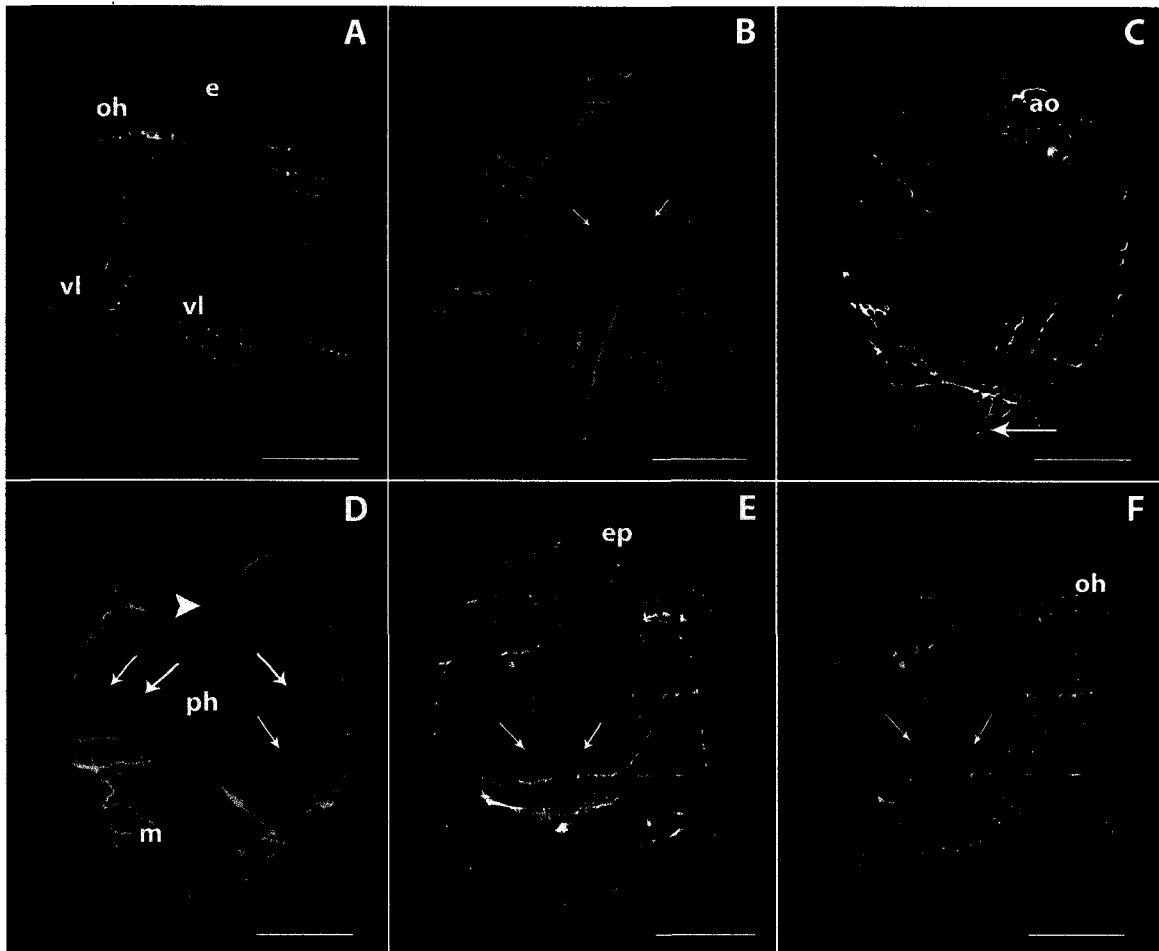


Figure 33. Larval stages of *Maritigrella crozieri*. (A) Ventral view of Müller's larva showing oral hood and two ventrolateral lobes, each with three longitudinal fibers. Small bright dots outlining the lobes represent the ciliary band; (B) dorsal view of the larva with longitudinal muscle fibers branching and forming small fibers (arrows); (C) dorsolateral view of the larva showing caudal region with five muscle fibers extending from dorsal to ventral surface (arrow); (D) deeper ventral view of the larva, arrows indicate inner ventrolateral muscles radiating from the oral rim to the lateral body wall. Arrowhead indicates two short longitudinal fibers crossing at the level of the oral hood; (E, F) lateral views showing lobe musculature extending from the distal end of the lobe to the apical end of the larva. Arrows indicate two groups of concentric muscles arranged in a lattice-like pattern. ao: apical organ, e: eyes, ep: epidermis, m: mouth, oh: oral hood, ph: rudimentary tubular pharynx, vl: ventrolateral lobe. All scale bars, 50µm.

During the next stage (61-71% of development, 102-120h, day 5), the embryos elongated and the larval shapes became clearly recognizable. At that time, the larval lobes, the oral hood, and the ciliary bands were evident (Figs. 33A, 34A). Ciliary bands

outlining the larval lobes could be recognized because actin in the microvillar collars surrounding the sensory cells (Lacalli 1982) stained intensely. Furthermore, rhabdite glands (actin staining in gland necks) became evident. This change in morphology was associated with the growth of many additional circular muscle bands and with the development of a few longitudinal fibers. The circular muscles became evenly spaced and completely encircled the embryo along its entire body length (Figs. 33A-C). Continuous rotations and strong uncoordinated contractions were observed during this stage. With the exception of the oral hood, which contained only circular fibers, all lobes contained three or four longitudinal muscle fibers. Within each lobe, these fibers originated from a single point located at the distal end of each lobe. From there, they extended fan-like through the lobes and eventually ran through the larval body towards the apical end of the larva (Figs. 33A, E, F, 34A-B). Each of these muscle fibers had been formed by the union of three or four individual filaments.

Within the larval body, two main longitudinal bands were observed extending from the posterior end to about half the body length where they met with the curvature of the dorsolateral lobes. From this point on, the two main fibers branched, forming thinner and smaller fibers, two of which reached the apical pole (Figs. 33B, 34B). Next to each of these fibers, were two individual longitudinal fibers extending the entire length of the body and giving rise to small fibers of the lobes (Figs. 33B, 34B). In a more lateral view, weakly-developed, longitudinal fibers extended into the lobes and appeared to converge onto muscles deep within the lobes, most likely parenchymal muscles (Fig. 33C). The caudal region of the body contained five muscle fibers (Fig. 33C), which connected dorsally with a thick circular fiber and ventrally with the musculature of the mouth (Figs. 33D, 34C). These bands were partly responsible for producing the extension and contraction of the larva.

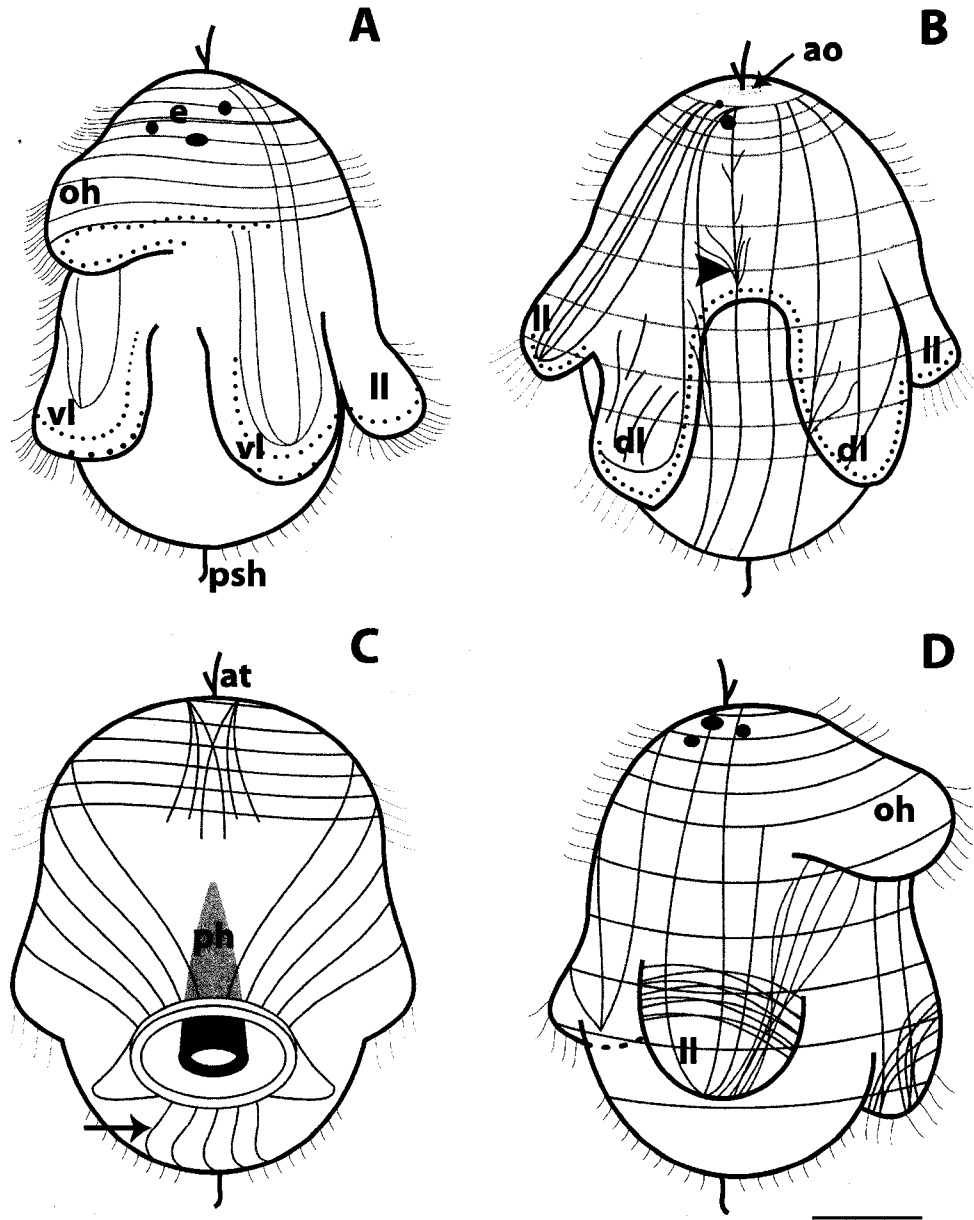


Figure 34. Schematic representations of the larval muscular system of *Maritigrella crozieri*. (A) Ventral view with circular fibers of the oral hood and longitudinal muscles of the ventrolateral lobes; (B) dorsal view with branching of longitudinal muscles at the junction of the dorsolateral lobes (arrowhead); (C) deeper view with ventrolateral muscles radiating from the oral rim. Arrow indicates caudal region with five muscle fibers connecting ventrally with the mouth. Short longitudinal muscles forming an X at the level of the oral hood. (D) lateral view showing two groups of concentric muscles crossing each other forming a lattice-like pattern in the lobes. ao: apical organ, at: apical tuft, dl: dorsal lobe, e: eyes, ll: lateral lobe, oh: oral hood, ph: rudimentary tubular pharynx, psh: posterior sensory hair, vl: ventrolateral lobe. Scale bar, 50 μ m.

The final stage ending with hatching, corresponded to 72-100% of development (121-168h, days 6-7). At this stage, the majority of the muscular system was formed and

only a few changes in the musculature of the lobes were observed. In the previous stage (61-71% of development, 102-120h), the inner musculature of the lobes was densely packed with thin, irregularly arranged filaments (Fig. 33C). During the final stage, these muscle fibers were extending in opposite directions, forming two groups of concentric muscles that overlapped each other and formed a diagonally arranged, lattice-like pattern (Figs. 33E-F, 34D). A distinct sphincter surrounded the ventrally located mouth, and an interior view of the larva, revealed individual ventrolateral muscles that radiated from the rim of the mouth and connected with the lateral body wall (Figs. 33D, 34C). Additionally, two short, longitudinal fibers crossed over each other at the level of the oral hood, forming a distinct X shape (Figs. 33D, 34C). At this stage, a tubular structure associated with the mouth was observed which corresponds to a rudimentary pharynx. At hatching, no diagonal muscle fibers were found and the entire arrangement of the muscles consisted of sets of circular and longitudinal fibers (Fig. 33F).

Muscle differentiation in *Melloplana ferruginea* (Table 1)

Embryogenesis in direct-developing polyclads generally takes longer than that of indirect-developing species. Furthermore, the eggs usually contain much larger amounts of yolk, a phenomenon that can slow development in the early stages. The eggs of *Melloplana ferruginea* were somewhat smaller than those of *Maritigrella crozieri* (on average 125 μm in diameter; n=50), and contained large amounts of yolk. This yolk emitted a strong autofluorescence (Fig. 35A). The period from oviposition to the beginning of gastrulation corresponds to approximately 0-15% development (0-79h). During early gastrulation (at about 14% development, 74h), the fluorescence started to abate and actin labeling became prominent and abundant all over the embryo (Fig. 35B).

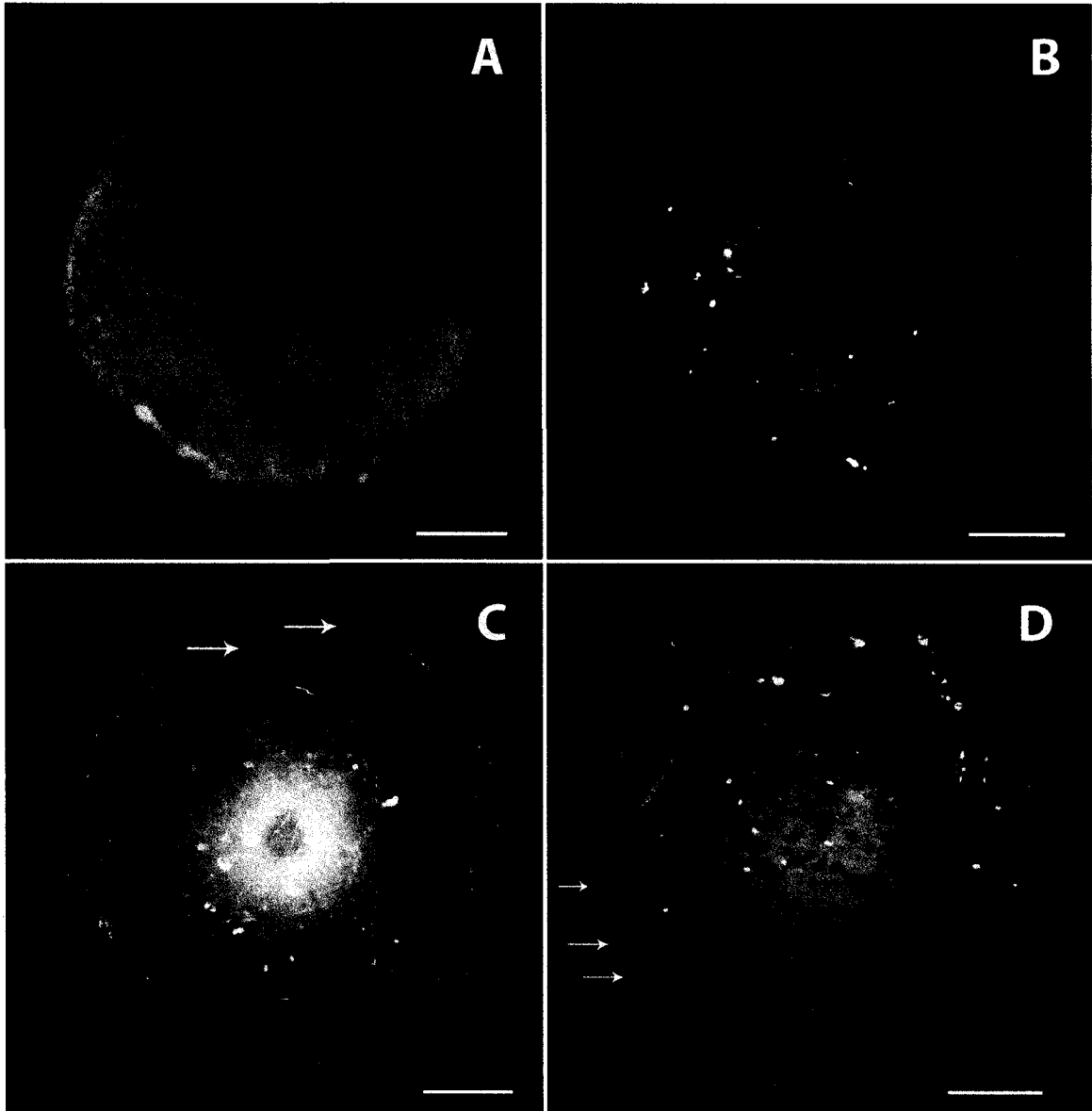


Figure 35. Muscle differentiation in *Melloplana ferruginea*. (A) Embryo after oviposition showing autofluorescence; (B) embryo at 15% of development with polygonal outlines of epidermal cells; (C) post-gastrulation embryo with bright spots of actin representing the anlagen of the body wall muscles. Arrows indicate short longitudinal muscle fibers in the periphery of the embryo; (D) yolk cells in the center of the embryo. Arrows indicate circular muscle fibers forming at the edges of the egg. All scale bars, 25µm.

Once gastrulation was initiated at about 15% of development (79h), the polygonal outlines of epidermal cells were visible (Fig. 35B) and continued to be distinct due to high concentrations of actin (16-21% of development, 84-111h, day 4). Moreover, a few bright spots of actin appeared within the embryo representing the anlagen of body

wall muscles (Fig. 35B). Autofluorescence still was present, and a few irregularly arranged muscle fibers were visible in the periphery of the embryo. At this stage (16-21% of development, 84-111h), first cilia appeared and minimal movement was evident.

At 22-30% development (116-152h, days 5-6), ciliation increased and slow rotational movements could be observed. Additionally, the bright muscle anlagen visible on the surface of the embryo increased in number and size (Fig. 35C). Although the fibers at the periphery of the embryo were still unorganized, a few myoblasts could be distinguished extending in circular and longitudinal directions. During the period of 31-44% of development (163-232h, days 7-9), the embryo was still spherical in shape and a single eye was present. The yolk cells migrated to the interior of the embryo diminishing the ability to visualize individual muscle fibers. Most myocytes appeared in an unorganized network. However, along the periphery of the embryo it was possible to differentiate some circular and diagonal fibers (Figs. 35C-D, 36).

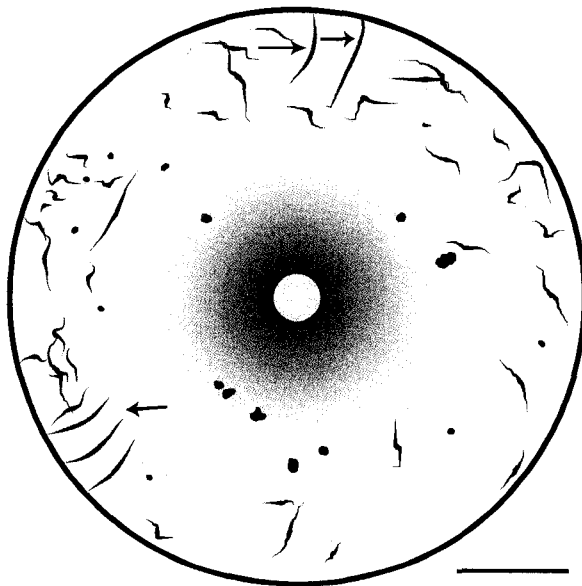


Figure 36. Schematic diagram of the egg of *Melloplana ferruginea* at 31-44% of development with unorganized muscle fibers in the periphery. Arrows indicate formation of longitudinal and circular muscle fibers. Scale bar, 25 μm .

A second small eye appeared at 45-59% of development (238-312h, days 10-13). In addition, faster rotations and first contractions of the juveniles were observed. The body elongated into a more ovoid shape, and the formation of a fine orthogonal grid

of circular and longitudinal muscles was evident (Figs. 37A, 39A). Unequally spaced circular fibers surrounded the entire embryo, whereas a few longitudinal fibers extended the length of the body, some of which bent diagonally at the anterior end of the worm. Circular fibers were better developed than the longitudinal muscle fibers but a more dense formation of longitudinal muscles was observed (Fig. 37A). Strongly-developed, spindle-shaped, dorsoventral muscles were visible extending across the body of the embryo (Figs. 37B-C). These muscles play an important role in locomotion and the maintenance of a flat body shape. Furthermore, parenchymal muscles were visible as unorganized fibers with a dense accumulation at the anterior end surrounding the brain anlage (Fig. 37B). During this period, the formation of the mouth on the ventral surface was visible as an elongated shape consisting of aggregated and unstructured muscle fibers (Fig. 37C).

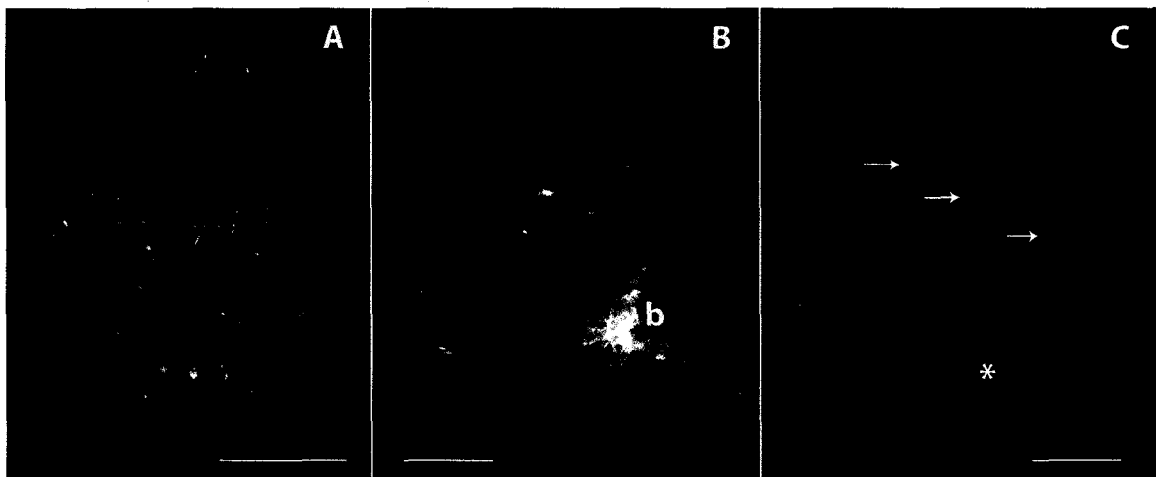


Figure 37. Embryos of *Melloplana ferruginea* at 45-59% of development. (A) Elongation of embryo showing a fine orthogonal grid of circular and longitudinal muscles. Scale bar, 50 μ m; (B) anterior end of embryo with an accumulation of parenchymal muscles surrounding the brain anlagen; (C) posterior end of the embryo with spindle shaped dorsoventral muscles traversing the body (arrows). Aggregation of unstructured muscle fibers forming the mouth on the ventral surface (asterisk). b: brain. Scale bars (B, C), 25 μ m.

At 60-71% of development (317-374h, days 14-16), the growth of diagonal muscle bands on the dorsal and ventral surfaces proceeded rapidly. Another pair of eyes

developed, and the mouth took on its circular shape (Figs. 38A-B, 39B). Dorsal, longitudinal muscle fibers extended straight from the anterior to the posterior end (Fig. 38A). Dorsally, a paired set of diagonal muscles covered the entire length of the worm, crossing over each other at a 90° angle (Figs. 38A, 39B). These fibers were denser and closer at the anterior part of the body becoming more spaced and still incomplete at the posterior end (Fig. 38A). Ventrally, diagonal muscle bands covered the anterior half of the body, crossing over each other at the body midline and then extending in a more longitudinal orientation along the lateral sides of the body (Figs. 38B, 39C). All diagonal muscle fibers were located between the longitudinal and the circular muscles.

The ventral musculature was more variable. Ventral, longitudinal fibers located along the body edges extended the entire length of the juveniles, but at the posterior end behind the mouth opening, they curved and formed U-shaped muscles (Figs. 38B, 39C). The ventral longitudinal muscle fibers close to the midline extended posteriorly in a straight fashion with some branching at the level of the mouth. An additional set of ventral longitudinal muscles was present running from the anterior end of the animal to about a third of the body length, where they bent laterally (Figs. 38B, 39C).

At this stage, parenchymal muscles were present throughout the body as disorganized, conspicuous, wavy fibers (Fig. 38C). Dorsally, long parenchymal muscles extended laterally to anchor at the body wall. Ventrally, shorter parenchymal fibers ran in varying directions. The concentric sphincter muscles of the mouth became established as a smaller, inner and a larger, outer ring of muscles (Figs. 38C-D). At this stage, short radial fibers seemed to connect the inner to the outer sphincter, however, they are most likely longitudinal muscles of the future pharynx (Fig. 38D). The ventral mouth was located at the very posterior end. Elongation of the growing embryo will cause the mouth to gradually move anterior to its final midventral position characteristic of adult

Melloplana ferruginea. Rhabdites could be distinguished and were more abundant on

the dorsal surface. Yolk droplets were still present in large amounts. The brain was well developed at this point.

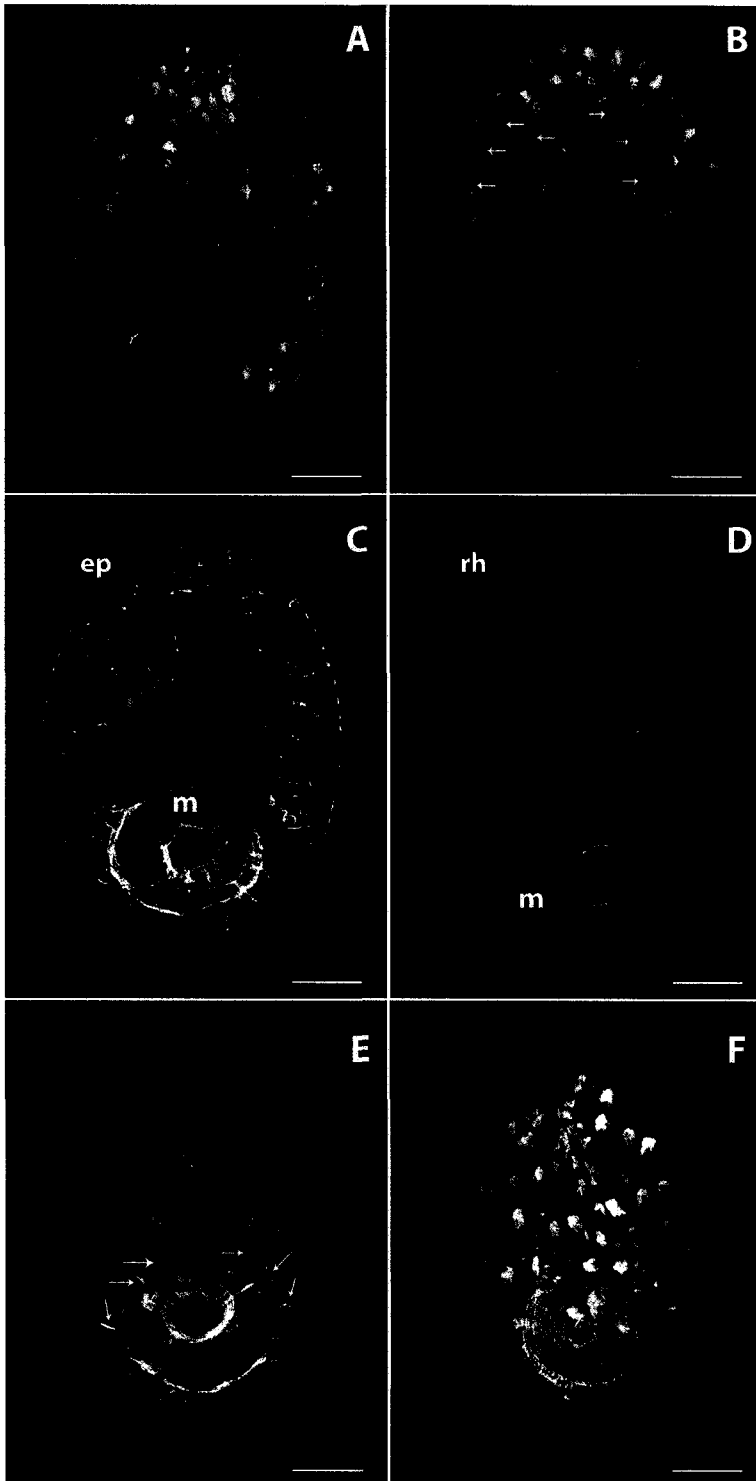


Figure 38. Musculature of *Melloplana ferruginea* embryos. (A) Dorsal musculature showing orthogonal pattern of longitudinal, circular and diagonal fibers; (B) dorsal musculature showing diagonal muscles enveloping the anterior half of the body (arrows); (C) irregularly arranged parenchymal muscles and oral musculature; (D) dorsal view of the mouth with concentric and longitudinal fibers; (E) complex musculature of the mouth forming a funnel-like structure. Arrows indicate oral dilator muscles; (F) dorsal surface with abundant rhabdites. ep: epidermis, m: mouth, rh: rhabdites. All scale bars, 25 μ m.

The final stage corresponds to 72-100% of development (380-528h, days 18-22). At approximately 78% of development (411h, day 17), the juveniles had almost completed their development but remained in their egg capsules for a few additional days, continuously rotating and contracting until hatching. Their bodies elongated and tapered posteriorly, acquiring their typical worm shape. During that period, only a few changes in the musculature were observed. The ventral parenchymal muscles and the oral musculature became more defined (Figs. 38E-F). No changes were observed in the body wall musculature.

The oral musculature is complex, formed by distinct sets of muscles (Figs. 38F, 39D): concentrically arranged muscles form a larger sphincter at the ventral surface of the worm and surround the mouth opening. The mouth opening invaginates into the body wall, leading into a funnel-like structure with a second, smaller sphincter located where the funnel narrows to a tube. The second sphincter is also composed of circularly arranged muscles. The funnel and tube are lined by longitudinal muscles, which appear to be arranged as radial muscles between the two sphincters because they are splayed outwards (Figs. 38E, 39D). Finally, distinct dilator muscles extending from both sphincters to the lateral body wall anchor the mouth (Figs. 38E, 39D). Random ventral parenchymal muscles that tended to extend in a more longitudinal direction, eventually will attach to the ventral and dorsal body wall and to organs. Other parenchymal fibers will remain suspended as individual fibers throughout the parenchyma. During this stage, an increase in the number of rhabdites was also observed (Fig. 38F).

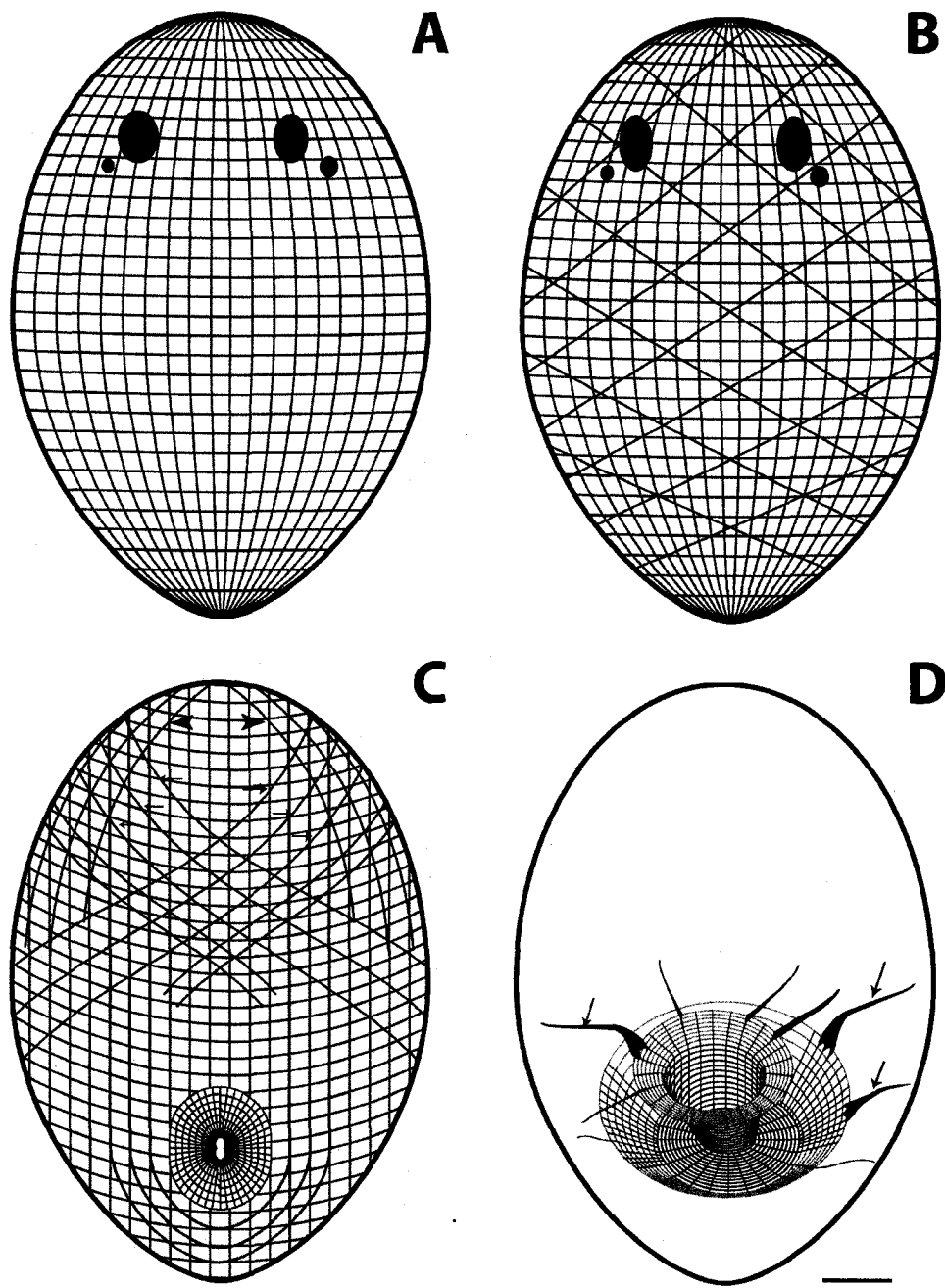


Figure 39. Schematic representation of the musculature in embryos of *Melloplana ferruginea*. (A) Dorsal surface with an orthogonal grid of circular and longitudinal muscles and two pairs of eyes; (B) dorsal surface with one set of diagonal muscles enveloping the entire length of the embryo; (C) ventral view with diagonal muscles (arrows) crossing each other at the body midline and extending along the lateral sides of the embryo. Arrowheads indicate a second group of ventral longitudinal muscles extending from the anterior end to about one third of the body length and bending laterally; (D) complex musculature of the mouth with dilator muscles (arrows). Scale bar, 25µm.

Discussion

In both species examined, the first myoblasts were localized in the periphery soon after epiboly. Cell lineage tracing studies in the indirect-developing polyclad, *Hoploplana inquilina* have shown that larval myoblasts derive from the 2b and 4d cells (Boyer et al. 1996, 1998). Such a dual origin of mesoderm has also been shown for the larvae of another polyclad, *Imogine mcgrathi* (Younossi-Hartenstein & Hartenstein 2000).

Progressively, the myoblasts formed unorganized and rudimentary muscle fibers that appeared at about 36% and 25% of development in *Maritigrella crozieri* and *Melloplana ferruginea*, respectively. Up to this point, muscle differentiation was similar between the two species; however, further muscle development progressed quite differently in larvae and juveniles. In *M. crozieri*, a primary circular muscle band appeared at about 40% of development. Later during development, this primary circular muscle band extended along the rim of the oral hood, a phenomenon also described for *Hoploplana inquilina* (Reiter et al. 1996). Development of a primary longitudinal muscle occurred at about 43% of development, at which time, additional circular fibers along the larval body had already formed. Because of large amounts of yolk granules obscuring events in *M. ferruginea*, it was not possible to time the earliest appearance of circular and longitudinal muscle fibers in this species. Instead, short circular and longitudinal fibers became evident simultaneously at about 45% of development. In both species, the orthogonal muscle grid of circular and longitudinal muscles was complete at the time when body elongation occurred (52% of development in *M. crozieri*; 59% of development in *M. ferruginea*).

Our results are comparable to muscle differentiation of *Imogine mcgrathi* (Younossi-Hartenstein & Hartenstein 2000) and *Macrostomum hystricinum marinum*

(Reiter et al. 1996) in which myoblasts formed circular and longitudinal fibers at approximately 60% of development. They differ though from the time line observed in *Hoploplana inquilina*, in which the first recognizable fibers appeared only at 80% of development while the embryo was still ovoid and had not yet acquired a typical larval form (Reiter et al. 1996). In comparison, 80% of development in *M. crozieri* corresponds to a clearly distinguishable larva with well-developed larval lobes containing muscle fibers and a recognizable body wall musculature. A case could be made that as an acotylean polyclad, the eggs of *H. inquilina* contain large amounts of yolk, which potentially could delay the growth of the myoblasts and the production of muscle-specific myosin. However, *I. mcgrathi* also is classified as an acotylean and its time line follows the one observed in *M. crozieri* (Younossi-Hartenstein & Hartenstein 2000).

Polyclads also possess a layer of diagonal muscles (Hyman 1951; Prudhoe 1985, Hooge 2001). In species with a larval stage, the diagonal muscles emerge only post-hatching as a result of larval metamorphosis (Reiter et al. 1996). We confirm this notion for specimens of *M. ferruginea*, in which diagonal muscles appeared at about 60% development and were well-developed by 70% developmental time. Hence, juveniles of direct developing species hatched with a body wall musculature characteristic for adults of the order. Contraction of diagonal muscles causes bending of the worms, a motion that is observed in juveniles even while still in the egg capsules. Larvae of *M. crozieri* on the other hand, did not have any diagonal muscles, possibly because bending motions are not required.

Larval lobes are adaptations for the planktonic phase of the animal. They are covered by long cilia that establish locomotory and feeding currents (Ruppert 1978). The musculature of the lobes is highly modified and upon contraction, will rapidly shorten the larva to an almost spherical shape. These myocytes provide body volume to the larva, and upon metamorphosis, some will become part of the body wall musculature, whereas

others will attach to organs or remain undifferentiated myocytes immersed in parenchymal tissue (Rieger et al. 1991a).

Parenchymal myocytes were observed as weakly staining fibers throughout the interiors of both embryos. They are located in areas of future organs and play a major role in the structure of the body. Because flatworms are soft-bodied animals, parenchymal muscles may contribute to skeletal support, in addition to being responsible for certain types of movements (Conn 1993). In juveniles of *Melloplana ferruginea* the spindle-shaped, dorsoventral muscles were well developed and were responsible for the flattened shape of the worms.

According to Reiter et al. (1996), the appearance of the first two longitudinal muscle bands parallel to the nerve cords during myogenesis of *Hoploplana inquilina* suggests a close relationship between the muscular and nervous systems during development. Similarly, in *Macrostomum hystricinum marinum* early myoblasts are located adjacent to the main nerve cords (Rieger et al. 1994). Although we did not specifically investigate such an association, it is reasonable to suppose that such a relationship also exists in the species examined in this study. Circumstantial evidence can be gleaned from *Melloplana ferruginea* where undifferentiated myoblasts surrounded the primitive brain.

The mouth is located ventrally as an invagination of the body wall. However, there were marked differences in mouth development between the two species. In *Maritigrella crozieri* the mouth opening was surrounded by a well-developed circular muscle consisting of about three concentric fibers and was connected with ventrolateral longitudinal muscles. An inner small muscular ring was associated with a tubular structure corresponding to a simple, straight pharynx. Pharyngeal muscles were not yet developed at the larval stage. According to Ruppert (1978), the development of a muscular pharynx occurs during larval metamorphosis. Hence, the lack of a pharyngeal

musculature in *M. crozieri* reflects the planktonic life style of a larval filter feeder. The same has been observed in the larvae of *Imogine mcgrathi* (Younossi-Hartenstein & Hartenstein 2000). In *Melloplana ferruginea* on the other hand, the mouth is associated with a more developed but still rudimentary pharynx. Two rings of circular muscles act as sphincters and are connected to dilator muscles, which in turn connect to the body wall. The mouth leads into a tubular structure lined by longitudinal muscles. Because of a funnel-shaped eversion of the mouth between the two circular sphincters, these longitudinal muscles appear to be radially arranged. However, this is the result of splaying out the oral opening. Pharyngeal musculature is important in structuring the shape of the adult pharynx, i. e., ruffled vs. tubular pharynx. This further differentiation is of taxonomic importance and is used for familial distinctions within polyclads (Faubel 1983, 1984; Prudhoe 1985).

A comparison of the body wall musculature of *Melloplana ferruginea* to that of Catenulida (Sterrer & Rieger 1974; Rieger et al. 1991b, Hooge 2001), other Rhabditophora (Hartenstein & Ehlers 2000), and Acoelomorpha (Tyler & Hyra 1998; Hooge 2001) reveals the same basic orthogonal pattern of circular and longitudinal muscles (with some arching of fibers around the mouth opening). Although the phylogenetic position of Acoelomorpha is still unresolved (Ruiz-Trillo et al. 1999, Jondelius et al. 2001; Wallberg et al. 2007; Deutsch 2008), their previous affinity with Platyhelminthes warrants such a comparison. According to Tyler & Hyra (1998), cylindrically elongated acoel species are characterized by the grid-like pattern, whereas species that are teardrop-shaped have more complex patterns with ventral longitudinal muscle fibers bending diagonally across the posterior part of the worm. Embryonic myogenesis has been described for the acoel, *Convoluta pulchra* (Ladurner & Rieger, 2000). Primary circular muscle fibers develop followed by the appearance of longitudinal fibers, resulting in the establishment of a typical muscular orthogon. Upon the

appearance of the mouth, the ventral longitudinal musculature is modified to extend medially and eventually cross to opposite side just posterior to the mouth. The same arrangement has been observed during regenerative myogenesis of the acoel *Convolutiloba longifissura* (Gschwenter et al. 2003). In the free-living rhabditophoran, *Mesostoma lingua*, embryonic myogenesis also results in a typical grid-like pattern of circular and longitudinal muscles (Hartenstein & Ehlers 2000). However, in the triclad, *Schmidtea polychroa* layers other than the orthogon (second longitudinal, diagonal) form during development and become part of the body wall musculature (Cardona et al. 2005).

A basic muscle orthogon is also the result of myogenesis in other Spiralia, e. g., nemertines (Turbeville 1991), many annelids (Hill 2001; McDougall et al. 2006; Bergter et al. 2007; although see Bergter et al. 2008 for a variation on the arrangement of longitudinal muscles in a small polychaete), mollusks (Haszprunar & Wanninger 2000; Wanninger & Haszprunar 2002) and sipunculids (Wanninger et al. 2005).

Although Polycladida clearly belongs to the Spiralia based on its cleavage pattern and dual origin of mesoderm (Boyer & Henry 1998; Boyer et al. 1996, 1998), a comparison of muscle development of larval *Maritigrella crozieri* with that of other larvae in the clade is dependent on the homology between polyclad larvae and trochophores. Whereas homology among trochophores of mollusks, annelids, nemertines, and sipunculids has been established (Nielsen 2001; Maslakova et al. 2004; Wanninger et al. 2005), homology of polyclad larvae with lophotrochozoan larvae has not yet been demonstrated. With this caveat in mind, we here compare the larval musculature of *M. crozieri* to that of other spiralian. Unlike the weakly-developed (or even absent) circular muscles bands in polychaetes where muscle formation may be controlled by segmentation (Bergter et al. 2008), most circular muscles of *M. crozieri* appear at the same time. This is comparable to the process observed in the sipunculid *Phascolion*

strombus in which circular muscles also appear simultaneously, not sequentially (Wanninger et al. 2005). Similarly, all repetitive dorso-ventral muscles of two species of chiton also appear simultaneously (Wanninger & Haszprunar 2002). This may be an indication of a closer relationship between unsegmented lophotrochozoans and polyclad flatworms.

As a basal lineage within Rhabditophora and as an order containing both indirect and direct developing species, a thorough understanding of muscle development in Polycladida has implications for existing models of the origin of body wall musculature of vermiform bilaterians. With the present study, we have added information not only from another indirect developing polyclad but also from a direct developing species. Our results provide additional support that the orthogonal muscle pattern is a symplesiomorphy of Spiralia and it may have been present in the stem species of all Bilateria (Ladurner & Rieger 2000; Rieger & Ladurner 2001, 2003).

Table 8. Comparison of embryonic myogenesis of *Maritigrella crozieri* and *Melloplana ferruginea*. Percentages refer to percentage of total developmental time (100% represents the time from oviposition to hatching). Asterisks indicate that the event was obscured by large amounts of yolk and could not be timed.

	<i>M. crozieri</i>	<i>M. ferruginea</i>
Epithelial polygons visible	30%	15%
Random myoblasts appear	36%	22-30%
Primary circular muscle bands	37-43%	*
Primary longitudinal muscle bands	43%	*
Circular musculature of larval lobes	44%	not applicable
Orthogonal muscle pattern established	45-52%	45-59%
Spindle-shaped dorsoventral muscles, unorganized parenchymal muscles	not applicable	45-59%
Development of oral musculature	53-60%	60-71%
Development of diagonal muscles	not applicable	60-71%
Longitudinal musculature of larval lobes	61-71%	not applicable
Orthogonal muscle pattern in larval lobes	72-100%	not applicable
Oral musculature established	72%	72%

Conclusions and Future Work

In this dissertation, I examined the embryonic development of several species of polyclad flatworms. My results show that there are clear morphological differences occurring during embryogenesis within the group. Although it is not possible to correlate the results with the current classification system, some of the data establish phylogenetic significance for each suborder. These results showed that different developmental patterns may be present in the group as adaptation for survival and dispersal; for example the case of poecilogony of *Pericelis cata*. Likewise, the observed modification of having extra-zygotic yolk may represent a special form of lecithotrophy for the larvae.

This dissertation also identified species suitable for experimental investigations (i.e., abundant, easy egg collection and maintenance under laboratory conditions, short developmental period). Classic cell lineage tracing studies using fluorescent dyes have been applied to only one indirect developing species in the suborder Acotylea. Hence, additional cell lineage tracing studies for further species, especially those with direct or intracapsular development, are desirable. Polyclad embryos are difficult to manipulate and culture under laboratory conditions. Moreover, some of the macromolecular tracers, species-specific genetic markers, or molecular probes that have been successfully employed in diverse groups of invertebrates are not available for polyclads or for flatworms in general. However, new techniques are being developed to facilitate the handling of small embryos. For example, whole-mount *in situ* hybridization has become the standard procedure for determining transcript expression patterns during embryonic development of other invertebrates, and it is likely that it will prove to be useful for polyclad embryos, too.

Furthermore, understanding the development of the muscular system has bearings for the understanding the origin of the body-wall musculature in the ancestor of

the Bilateria. Data on myogenesis in an early lineage such as the Polycladida, may support the hypothesis that the orthogonal muscle grid appeared very early during the evolution of the muscular system, perhaps even in a diploblastic ancestor. Again, additional experimental studies regarding the evolution of germ layers will elucidate the transition from diploblastic animals to triploblastic Spiralia. Finally, the close interplay between the muscular and nervous systems during development warrants further investigation.

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APPENDICES

APPENDIX A

A NEW ACOTYLEAN FLATWORM, *Armatoplana colombiana* n. sp.
(PLATYHELMINTHES: POLYCLADIDA: STYLOCHOPLANIDAE) FROM THE
CARIBBEAN COAST OF COLOMBIA, SOUTH AMERICA¹

Introduction

The order Polycladida represents a highly diverse clade of free-living, almost exclusively marine flatworms (Prudhoe 1985). Polyclads are found from the littoral to the sublittoral zone; on coral reefs, among shells and seaweeds, as well as on colonial ascidians.

Based on the character presence/absence of a cotyl or sucker, Lang (1884) divided the order into the Cotylea and Acotylea, respectively. Of the two, the Acotylea is the larger group with over 28 families worldwide. Most acotyleans are dull in coloration, negatively phototactic and cryptic in their behavior, hiding in crevices and under coral during the day. Many acotyleans are major predators of commercial bivalves (Galleni et al. 1980, Prudhoe 1985, Littlewood & Marsbe 1990, Newman et al. 1993, Jennings & Newman 1996a, b, O'Connor & Newman 2001).

Despite the fact that many polyclads pose a threat to aquaculture industries, acotyleans and polyclads in general, have received little taxonomic attention. This is due to difficulties in collecting, preserving, and identifying specimens. Polyclads are known to autolyse upon handling, and it was not until Newman & Cannon (1995) developed a new

¹ Bolaños, DM., Quiroga, S. & Litvaitis M.K. 2006. A new acotylean flatworm, *Armatoplana colombiana* n. sp. (Platyhelminthes: Polycladida: Stylochoplanidae) from the Caribbean coast of Colombia, South America. *Zootaxa* 1162: 53-64

fixation technique, that routine distortion could be avoided during histological processing. In addition, positive species identification of acotyleans requires serial sagittal sectioning of the reproductive system, a process that is time-consuming and reliant on expert knowledge. Thus, it is not surprising that polyclads have largely been ignored, and that only scant information is available on their distributions.

The earliest surveys of polyclads in the Caribbean include those of Prudhoe (1944) in the Cayman Islands, Hyman (1939, 1955a, b) in the US Virgin Islands, Jamaica, Puerto Rico, Bermuda, the Bahamas, Dominica, and Florida, and Marcus and Marcus (1968) in the Lesser Antilles, Puerto Rico, Key Biscayne, and Brazil. More recently, in a survey of the Tayrona National Park in Colombia, Quiroga et al (2004) listed 25 species of Polycladida. Of these, 13 species belong to the Acotylea, bringing the total number of acotyleans recorded for the Caribbean to 78 species.

In this account, we describe a new species of the genus *Armatoplana* Faubel 1983 from the Caribbean coast of Colombia. This species was previously listed as an undescribed species of *Pleioplana* Faubel 1983 (Bolaños et al. 2004, Quiroga et al. 2004).

Material and methods

Polyclads were hand collected in the littoral zone from under rocks at Inca-Inca in Gaira Bay, Santa Marta, Colombia. Animals were measured (measurements given as length mm x width mm) and photographed *in vivo* in the lab, fixed on frozen 10% buffered formalin and preserved in 70 % ethanol. From one specimen, a 3mm x 2 mm segment was dissected containing the reproductive structures. This segment was embedded in paraffin, sagittally sectioned at 5–7 μ m, and stained with hematoxylin and eosin. Sections were mounted in Permount on glass slides. Diagrammatic reconstructions of the reproductive system were derived from the sectioned material.

The material has been accessed into the Museo de Historia Natural Marina de Colombia at INVEMAR in Santa Marta as a wet specimen and as serial sections. Taxonomic identifications were done following the classification system of Faubel (1983), which is based on the characteristics of the male reproductive system, specifically the structure of the prostatic vesicle and its orientation and relationship to the ejaculatory duct.

Systematics

Family: Stylochoplanidae Faubel 1983

Genus: *Armatoplana* Faubel 1983

***Armatoplana colombiana* n. sp.**

(Figs. 1–9)

Type material

Holotype, one mature specimen (5.5 mm X 3 mm) in 70% ethanol, INV-PLA 0019; collected in August 2002.

Paratype, one mature specimen (6 mm x 2.5 mm) as serial sagittal sections, INV-PLA 0020 HS, collected in August 2002.

Other Material Examined: one additional mature specimen (6 mm x 3 mm); reproductive system sectioned sagittally.

Type Locality: Inca-Inca (N11° 11'; W74° 14'), Gaira Bay, 6 km southeast of Santa Marta, Colombia.

Etymology

Species name refers to Colombia, the country from which the type specimens were collected.

Synonyms

Pleioplana sp. Bolaños et al., 2004. *Pleioplana* sp. Quiroga et al., 2004.

Distribution

To date, found only at Inca-Inca Bay, Tayrona National Park, Santa Marta, Colombia, from under rocks in the littoral zone.

Diagnosis

Species characterized by non-retractile nuchal tentacles and by 6–8 submarginal knobs at the anterior end. Male stylet extremely long (1250–1500 μm), curved, with very pointed end.

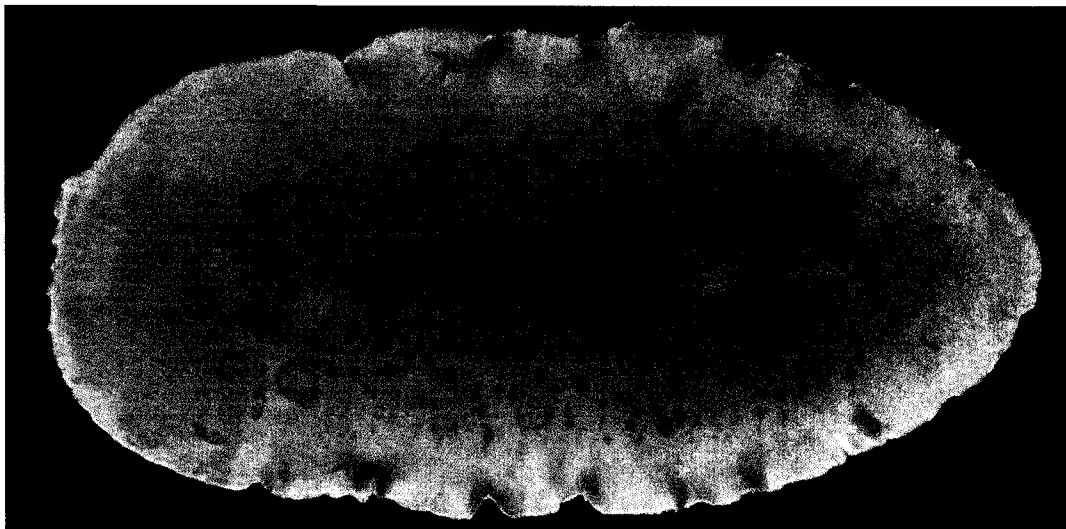


Figure 1. Whole, fixed animal, representing general body shape and nuchal tentacles.

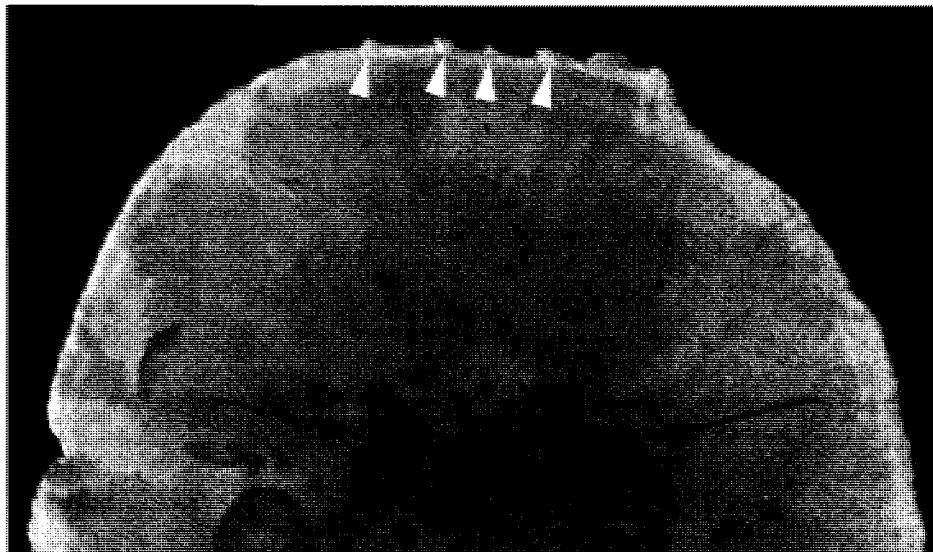


Figure 2. Fleshy, anteriorly located knobs (white arrow heads) and nuchal tentacles (black arrows).

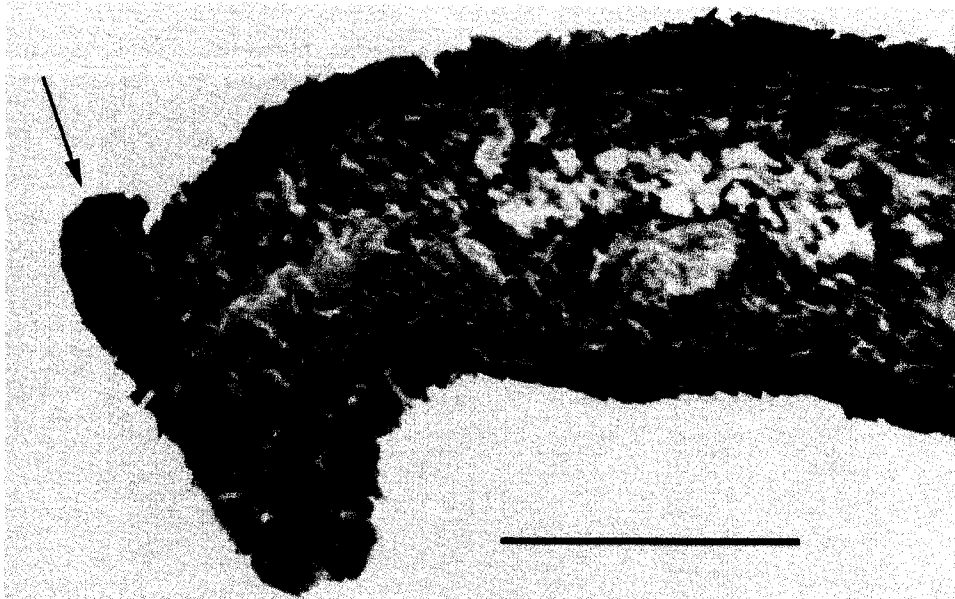


Figure 3. Sagittal section through the anterior end, indicating position of knobs (arrow); the anterior end is folded subterminally. Scale = 1 mm.

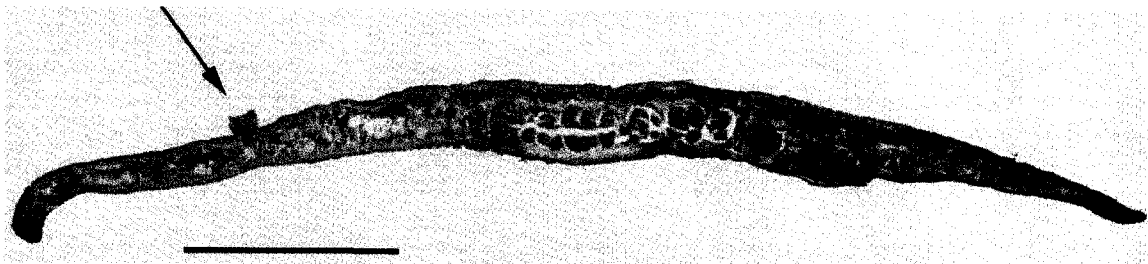


Figure 4. Sagittal section with nuchal tentacles (arrows). Scale = 1 mm.

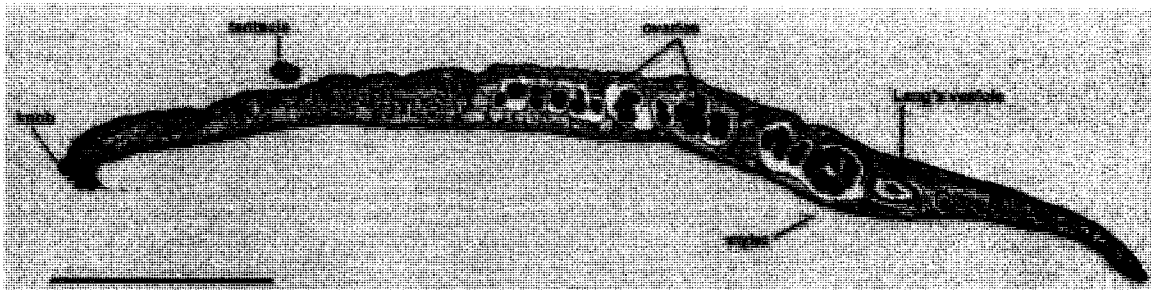


Figure 5. Sagittal section showing one anterior knob, tentacle, ovaries, Lang's vesicle, and distal point of extruded stylet. Scale = 1 mm.

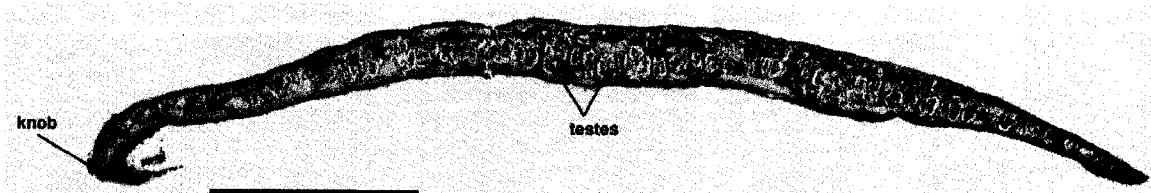


Figure 6. Sagittal section showing ventral location of testes. Scale = 1 mm.

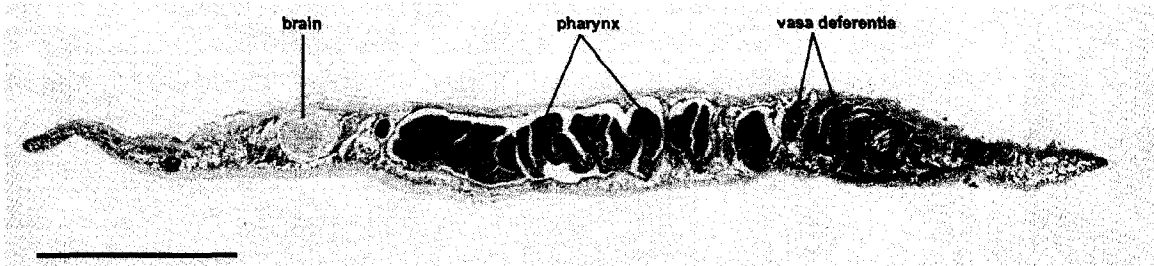


Figure 7. Sagittal section displaying anterior brain capsule, voluminous pharynx, and highly sinuous vasa deferentia. Scale = 1 mm.

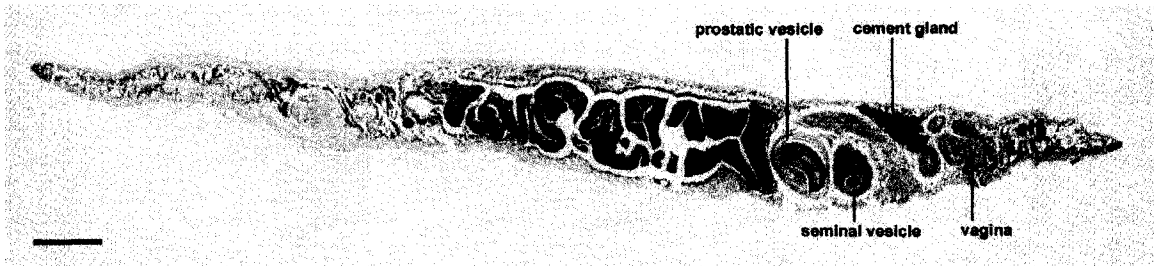


Figure 8. Sagittal section showing prostatic and seminal vesicles, sinuous vagina, and cement glands. Scale = 250 μ m.

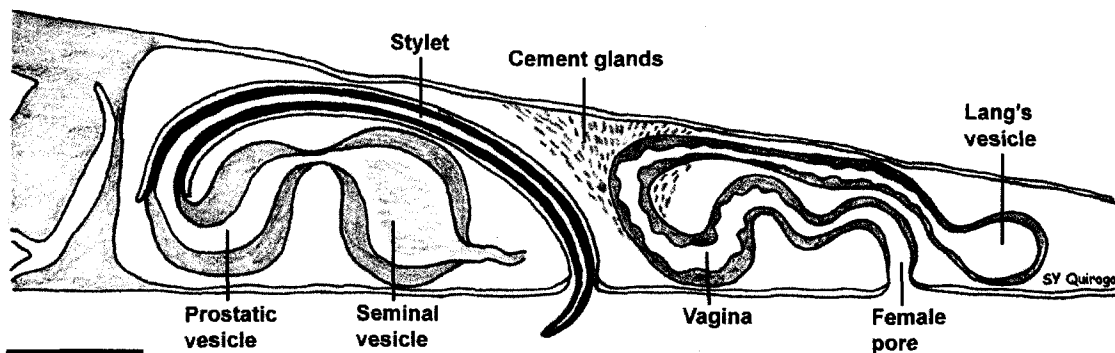


Figure 9. Diagrammatic representation of copulatory complex of *A. colombiana*. Scale = 250 μ m.

Description

External features: Small worms, of light grayish color, with dorsal surface covered with an irregular distribution of brown spots (Fig. 1). Anterior end rounded and bearing 6–8 fleshy, well-separated knobs (Figs. 2, 3, and 5). Short (200 μ m long), non-retractile nuchal tentacles present just lateral to the brain (Figs. 2, 4 and 5). Small eyes scattered at the base of the tentacles and in the cerebral region as three eye clusters. Ruffled pharynx centrally located in anterior third of body, mouth at posterior end of pharynx.

Uteri visible through body wall, running anterior, anastomosing just above the anterior end of the pharynx. Male and female gonopores separate and posterior to pharynx. Anterior and posterior heavy concentration of rhabdites in epidermis. Posterior end pointed.

Reproductive anatomy: Measurements refer to lengths in a 4.3 mm long worm. Male copulatory apparatus located anterior to male pore and directed posteriorly. Very deep, male antrum houses a long (1250 x 50 μm) and curved stylet, stylet curves dorsally over the seminal and prostatic vesicles. In the majority of fixed worms, the stylet is extruded from the male pore (Fig. 5). Prostatic vesicle (275 x 225 μm) interpolated, seminal vesicle (275 x 175 μm) joined dorsally to prostatic vesicle (Fig. 9). Both seminal and prostatic vesicles with strongly muscularized walls. Prostatic and seminal vesicles very close to each other, hence it is very difficult to distinguish between them. Prostatic vesicle slightly curved, joined almost directly to the stylet. Testes ventral (Fig. 6); highly sinuous vasa deferentia (Fig. 7) joined dorsally to seminal vesicle. Female reproductive system with very sinuous vagina with ridged walls (Fig. 8); Lang's vesicle present. Uteri highly voluminous. Male gonopore close to female pore. A schematic representation of the reproductive complex is given in Fig. 9.

Taxonomic Remarks

Bock's (1913) seminal work on the Polycladida divided the Acotylea into three sections based mostly on the arrangement of the eyes, namely the Emprostommata, Craspedommata, and Schematommata. Prudhoe (1982) in turn, emended these divisions into three superfamilies, the Cestoplanoidea, Stylochoidea, and Planoceroidea, respectively. Using mostly characters of the male reproductive system, Faubel (1984) revised the three superfamilies to Ilyplanoidea (true prostatic vesicle lacking), the Stylochoidea (prostatic vesicle free), and the Leptoplanoidea (prostatic vesicle

interpolated), respectively (Table 1; see also Tyler et al. 2005). Within the Leptoplanoidea, Faubel (1984) established three new families, one of which, the Stylochoplanae, he validates with the characters “true prostatic vesicle present, its glandular lining smooth and the glands of which mostly extraventricular.”

Table 1. Comparison of the changing nomenclature of the three acotylean superfamilies.

Authority	Superfamily	Superfamily	Superfamily
Bock 1913	Craspedommata	Schematommata	Emprostommata
Poche 1926	Stylochoidea	Planoceroidea	Cestoplanoidea
Marcus & Marcus 1966	Craspedommatidea	Schematommatidea	Emprostommatidea
Prudhoe 1982	Stylochoidea	Planoceroidea	Cestoplanoidea
Faubel 1983	Craspedommatidea	Schematommatidea	Emprostommatidea
Faubel 1984	Stylochoidea	Leptoplanoidea	Ilyplanoidea
Tyler et al. (2005)	Stylochoidea	Leptoplanoidea	Ilyplanoidea

Within this family, the genus *Stylochoplana* (Stimpson 1857) consists of a heterogeneous assemblage of numerous species. Recognizing the need for a more appropriate classification, Marcus & Marcus (1968) had separated the genus into groups based on presence or absence of tentacles and of a stylet. Their group C2, characterized by the presence of tentacles and an armed penis, contains three species, *S. divae*, *S. vesiculata*, and *S. evelinae* (Marcus & Marcus 1968).

Since then, Faubel (1983) erected the genus *Armatoplana*, including those species of *Stylochoplana* characterized by an armed penis and the presence of Lang’s vesicle. Species with an unarmed conical penis papilla were retained in *Stylochoplana*. Faubel (1983) distinguishes species of *Armatoplana* from species in other genera of the family by the following combination of characters: lack of tentacles, presence of serial cerebral and tentacular eyes, an anteriorly located pharynx, presence of a true seminal vesicle or

spermiducal bulbs, and an armed penis with a long, sharp stylet. Lang's vesicle and a true vagina bulbosa are present in the female copulatory apparatus. However, the character "presence/absence of nuchal tentacles" may be of little systematic value, because Faubel (1983) moved two species with head tentacles, *S. divae* and *S. vesiculata* of group C2 (Marcus & Marcus 1968) into *Armatoplana*. Based on this, we believe it is appropriate to amend *Armatoplana* to include worms with or without nuchal tentacles. This is further supported by the fact that nuchal tentacles may be difficult to discern in poorly fixed material and may have been overlooked in the past.

With the exception of the presence of nuchal tentacles in our specimens, the newly described species *Armatoplana colombiana*, agrees with all the characteristics of the genus as defined by Faubel (1983). However, as stated above, the presence of tentacles may not be of great taxonomic significance. Initial identifications that had placed this species into the genus *Pleioplana* were based mostly on a general arrangement of reproductive structures and the presence of a long, pointed stylet (Bolaños et al. 2004, Quiroga et al. 2004). Since then, it has become clear that the prostatic vesicle of *Pleioplana*, containing well-defined tubular chambers, is very different from the prostatic vesicle observed in our specimens. The only other genus within the Stylochoplanidae that is characterized by an armed penis, the presence of Lang's vesicle and tentacles is *Interplana*. However, in species of *Interplana*, the stylet does not curve dorsally over the prostatic and seminal vesicles as it does in species of *Armatoplana*.

The presence of anterior marginal knobs (Figs. 2, 3 and 5) which are lacking in *A. divae* and *A. vesiculata*, clearly separates *A. colombiana* from these congeners, as do differences in reproductive system structures, i.e., *A. colombiana* has a much longer and more curved stylet, the prostatic vesicle is more rounded, and Lang's vesicle is bigger (Table 2). In addition, no mature specimens of *A. divae* and *A. vesiculata* are

known that are less than 1 cm in length. The shape and nature of the male reproductive systems of *A. lactoalba*, *A. leptalea*, and *A. panamensis* show close similarities with those of *A. colombiana*. However, based on the presence of tentacles and marginal anterior knobs in *A. colombiana*, the new species can be reliably separated from these three species (Table 2). Additionally, coloration and size can be used to differentiate among these species. Finally, live specimens of *A. colombiana* may be confused with *Styloplanocera fasciata* because coloration and color patterns (light grey with isolated dark brown spots and a network of brown pigmentation covering the dorsal surface) of the two are almost identical. However, a closer examination of *S. fasciata* will show that knobs are present all over its surface, whereas they are limited to the anterior end in *A. colombiana*. Additionally, *S. fasciata* of such a small size would not be mature individuals. Internally, of course, the male reproductive systems of *S. fasciata* and *A. colombiana* are completely different, again emphasizing the importance of histological sections for positive species identifications in Acotylea.

Table 2. Comparison of morphological features of existing valid and proposed new species of the genus *Armatoplana* (characters according to Faubel, 1983)

Species	Coloration	Sub-marginal knobs	Tentacles	Tentacular eyes	Cerebral eyes	Seminal vesicle	Prostatic vesicle	Penis stylet	Lang's vesicle
<i>A. colombiana</i> n.sp	greyish transparent, with brown spots distributed irregularly over dorsal surface	6-8 knobs present at anterior end	non-retractile nuchal tentacles	small eyes scattered at base of tentacles	three small clusters	muscular joined to prostatic vesicle dorsally	interpolated, muscular with slight curvature; located parallel to seminal vesicle	very long and curved	small, rounded
<i>A. affinis</i> (Palombi, 1940) Faubel 1983	yellow with brownish dots over dorsal surface	absent	absent	two groups, next and behind of cerebral eyes	one big, dense group	oval, muscular	oval, muscular	small, slender and long	oval, small with two spherical accessory vesicles
<i>A. divae</i> (Marcus, 1947) Faubel 1983	olive green with brown intestinal ramifications extending from middle to periphery	absent	short nuchal tentacles	one group at base and surrounding each tentacle	two small, dense groups	large, elongate below prostatic vesicle	interpolated; dorsal and parallel to seminal vesicle	Conic and long	small, rounded
<i>A. lactea</i> (Laidlaw, 1903) Faubel 1983	white with minute grey dots scattered sparsely over dorsal surface	absent	absent	two small groups with a few eyes	small group with few eyes wide spread over brain area	two elongated seminal vesicles with thick muscular walls	reduced	small, armed and curved	small, muscular
<i>A. lactoalba</i> (Verrill, 1900) Faubel 1983	translucent milky white	absent	absent	two longitudinal bands with numerous eyes enlarged at the level of the brain	absent	oval, large, very muscular	oval, chambered	long slender	large, long
<i>A. leptalea</i> (Marcus, 1947) Faubel 1983	translucent	absent	absent	two longitudinal bands of few eyes extended to pre-cerebral region	small group of few eyes	elongate, muscular, anterior to male gonopore	interpolated, anterior to seminal vesicle	muscular, slender and long	narrow, long
<i>A. panamensis</i> (Plehn, 1986) Faubel 1983	grey	absent	absent	two groups of about 12-15 eyes each, some large, others small	two groups with numerous eyes, not distinctly separated from tentacular groups	oval, muscular; joined directly to prostatic vesicle	long, narrow; directly joined to seminal vesicle	armed, long and curved	extraordinary large, elongated

<i>A. rabita</i> (Marcus & Marcus, 1968) Faubel 1983	light yellow with two longitudinal chestnut brown bands	absent	absent	absent	two forward converging rows but only a few distinct eyes	small group of tiny eyes directed dorsally and ventrally	small, oval; anterior to prostatic vesicle	interpolated, large, elongated; posterior to seminal vesicle	short and conical	short, rounded
<i>A. reishi</i> (Hyman, 1959) Faubel 1983	Pale	absent	absent	absent	definite clusters of about six eyes in each group	one group of 18-20 eyes extending forward linearly	fusiform, muscular below prostatic vesicle	oval; above seminal vesicle	conical penis papilla and long stylet	small, crescent shape
<i>A. robusta</i> (Palombi, 1928) Faubel 1983	dark chestnut brown	absent	absent	absent	two groups, large and of irregular form	two small groups with numerous eyes irregularly arranged	sac-shaped, directly joined to prostatic vesicle	elongate; directly joined to seminal vesicle	short	small, short, extending forward
<i>A. snadda</i> (Marcus & Marcus, 1968) Faubel 1983	unknown	absent	absent	absent	two dense clusters directed peripherally	one group anterior and medial of brain, extending anteriorly; plus single cerebral eye behind each tentacular cluster	large, circular, muscular; below prostatic vesicle	small, rounded above seminal vesicle	slender and long	small, moniliform
<i>A. taurica</i> (Jacubova 1909) Faubel 1983	unknown	absent	absent	present	two dense groups covering tentacles	two long rows with few eyes extending anteriorly	small, elongate, muscular, directly joined to prostatic vesicle	long, elongate, posterior to seminal vesicle	short, small	small, rounded
<i>A. tenuis</i> (Palombi, 1936) Faubel 1983	dark brown	absent	absent	absent	two dense rows extending beyond brain level	one irregularly arranged group not distinctly separate from tentacular groups	elongate, directly joined to prostatic vesicle	small, oval, elongate; bent towards posterior end	small, conic penis without stylet	small, rounded
<i>A. vesiculata</i> (Palombi, 1940) Faubel 1983	grey and yellow, clearer in the pharynx area	absent	absent	absent	two groups	two long, dense groups in middle of tentacular eyes	small, elongate, muscular	piriform	small penis with strong stylet	elliptical, large with two spherical accessory vesicles

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APPENDIX B

FIVE NEW SPECIES OF COTYLEAN FLATWORMS (PLATYHELMINTHES: POLYCLADIDA) FROM THE WIDER CARIBBEAN

Introduction

The polyclad family Pseudocerotidae includes some of the most conspicuously colored and extraordinarily diverse marine flatworms. They have been found throughout tropical and subtropical waters and are prominent members of coral reef communities. Currently, 13 genera are included in the family, with *Pseudoceros* and *Pseudobiceros* contributing the majority of species. In general, polyclad species are distinguished by characters of their reproductive systems (Faubel 1983, 1984). However, many pseudocerotids are known for their remarkably uniform reproductive anatomy and hence, species diagnoses are based primarily on coloration and color pattern (Hyman 1954, 1955a, b, 1959a, b; Prudhoe 1989; Newman & Cannon 1994, 1996, 1997, 1998). Faubel (1984) recognized that some species of *Pseudoceros* had two complete sets of male reproductive systems. This led him to erect the new genus *Pseudobiceros* and to transfer all *Pseudoceros* species with a double male system into this new genus (Faubel 1984). Since then, *Pseudobiceros* has been further validated by characters of pharynx type, arrangement of cerebral eyes, body margin ruffling, and molecular data (Newman & Cannon 1994, Litvaitis & Newman 2001, Rawlinson & Litvaitis, in press).

¹ Bolaños, DM., Quiroga, S. & Litvaitis M.K. 2007. Five new species of cotylean flatworms (Platyhelminthes: Polycladida) from the wider Caribbean. *Zootaxa* 1650: 1-23.

The genus *Thysanozoon* also belongs to the family Pseudocerotidae, and occasionally, species determinations can be made using coloration and color pattern as well. Additionally, two external features such as a papillated dorsal surface and the presence of two male gonopores allow for an easy placement of animals into this genus. Although species of *Acanthozoon* also show papillae on their dorsal surface, they only have one male gonopore and thus, can be readily distinguished from members of *Thysanozoon*. *Thysanozoon* is a circum-globally distributed and poorly studied genus. Risso (1818) was the first to describe two species but erroneously placed them into the molluscan genus *Tergipes*. Grube (1840) recognized Risso's two species as synonyms and established the genus *Thysanozoon* in the Pseudocerotidae.

An equally diverse and colorful family, the Euryleptidae also is an inhabitant of coral reefs and can easily be confused with the pseudocerotids, especially with species of *Pseudoceros*. A major distinction, though, is the presence of a tubular rather than a ruffled pharynx. Many of the genera in this family are very similar to each other and only few external and internal characters are distinct. The most recently described genus within the family is *Maritigrella* (Newman & Cannon 2000). The diagnosis for the genus includes an elongated body, marginal tentacles that are long and held erect, two elongated clusters of cerebral eyes, a small, muscular and tubular pharynx, a short, pointed and sclerotized stylet, no uterine vesicles, and an especially distinct striped color pattern (Newman & Cannon 2000).

Here we describe five new cotylean species collected from different localities in the Caribbean; four belonging to the Pseudocerotidae and one to the Euryleptidae. Additionally, we are re-describing *Pseudoceros pardalis*, Verrill 1900 and propose the new combination *Pseudobiceros pardalis*.

Material and Methods

Field Collection

Specimens were hand collected using a fine paint brush either in the littoral zone from under rocks and from under coral rubble or subtidally from coral reefs in Panama, Curaçao, Jamaica, the Florida Keys, Honduras, and the US Virgin Islands. Specific locations, habitat information, and georeferences are given with each species. Animals were measured (measurements given as length mm x width mm) and photographed *in vivo* in the lab.

Histology

Specimens were fixed on frozen 10% buffered formalin following the protocol developed by Newman & Cannon (1995). After fixation, animals were preserved in 70% ethanol for histological preparation. The portion of the animal containing the reproductive structures was dissected. This segment was embedded in paraffin, sagittally sectioned at 5–7 μm , and stained with Gill's hematoxylin and eosin. Sections were mounted in Permount on glass slides. For whole mounts, animals were dehydrated, cleared in HistoClear, and mounted in Histomount. Diagrammatic reconstructions of the reproductive system were derived from the sectioned material and from whole mounts.

Taxonomic identifications of *Pseudoceros*, *Pseudobiceros*, and *Maritigrella* were based on the descriptions of color and color patterns and for each species we include the color pattern grouping as defined by Newman & Cannon (1994, 2000). Further information from the classification system of Faubel (1984), which is based on characteristics of the male reproductive system, was used for the determination of *Thysanozoon*. Most type material has been deposited at the US Natural History Museum (USNM) in Washington, DC, USA. Specimens collected on St. John, US Virgin Islands have been deposited in the VIIS collection at the Biosphere Reserve Center on St. John.

Generation of Molecular Tags

From each specimen, we excised a small piece of tissue for DNA analysis. If the tissue piece was large enough, only part of it was used for DNA extraction. The remaining tissue samples can serve as future vouchers. High molecular weight DNA was extracted using Genomic Tips (Qiagen Inc., Valencia, CA). The target nucleotide sequence was the D1–D2 expansion segment of the 28S rDNA gene. Primer sequences (LSU D1, D2 fw1 and LSU D1, D2 rev2) can be found in Sonnenberg et al (2007). DNA amplifications followed the protocol outlined in Litvaitis & Newman (2001). Amplicons were gel-purified and sent to a commercial lab for sequencing (Geneway Research LLC, Hayward, CA). Sequencing occurred in both directions. Trace files were edited using FinchTV (vers. 1. 4; Geospiza Inc) and sequences were deposited in GenBank. Accession numbers are provided with individual species descriptions.

Systematics

Superfamily: Pseudocerotoidea Faubel, 1984

Family: Pseudocerotidae Lang, 1884

***Pseudobiceros pardalis* (Verrill, 1900) n. comb.**

(Figs. 1, 2)

Material Examined

Three specimens collected subtidally, Panama, Bocas del Toro, Salt Creek (N9° 16.806'; W82° 06.137') in July 2004.

a) One specimen (UNH-PAN 028, 40mm x 25mm) sagittally sectioned (18 slides); USNM 1104638. Collected 18 June, 2005 under *Acropora palmata* coral rubble, from the reef crest, 1.5 m depth.

b) One specimen (UNH-PAN 029, 38mm x 22mm) as whole mount (1 slide); USNM 1104639; GenBank Accession EF514807. Collected 18 June, 2005 under *Acropora palmata* coral rubble, from the reef crest, 2 m depth.

c) One specimen (UNH-PAN 056, 25mm x 14mm) as whole mount (1 slide); USNM 1104685; GenBank Accession EF514808. Collected 28 June, 2005 under *Acropora palmata* coral rubble, from the reef crest, 1.5 m depth.

Synonyms

Pseudoceros pardalis Verrill, 1900 (p. 596; plate LXX, Fig. 6a), see taxonomic remarks below.

Distribution

This species has been reported for Bermuda (Verrill 1900), the Bahamas, and southern Florida (Humann & DeLoach 1992); it was common in Bocas del Toro, Panama.

Diagnosis

Purple-brownish background blending into black towards the margin. Dorsal surface with large orange and smaller yellow dots outlined by a black shadow. Numerous, small white spots along the margin around the entire body. Light purple-violet ventral surface, more translucent towards the margin.

Re-description

External Features: Coloration as described above for diagnosis (Fig. 1A). In preserved specimens, the coloration is "brownish-black, covered with numerous round, pale yellow spots" (Verrill 1900). According to the color pattern groups established for *Pseudobiceros* (Newman & Cannon 1994), the species belongs to Group 4 (spots, dots and mottling). The body is large, oval shaped, and fleshy. Prominent pseudotentacles are formed by simple folds of the anterior margin (Fig. 1A). The cerebral eyespot is oval with about 90–100 eyes. A slightly ruffled pharynx is located in

the anterior one-third of the body and the mouth is a large opening located at the anterior end of the pharynx. The sucker is conspicuous and centrally located (Fig. 2D).

Body Wall: The epidermis is of uniform thickness dorsally and ventrally. It consists of a simple, ciliated epithelium that surrounds the entire body. Epithelial cells are closely aggregated, making it difficult to differentiate the nuclei. Rhabdite glands are more abundant on the dorsal side. However, they are very numerous around the sucker and gonopores on the ventral side. Loose connective tissue is irregularly distributed dorsally but forms a fine network with considerable extracellular spaces ventrally, supporting the seminal and prostatic vesicles and the sucker. The basement membrane is extremely thin. The body wall musculature is weakly developed with thin muscle fibers. Color is imparted endogenously (i. e., not due to ingested food) by dark pigment granules located in dense, oval clusters beneath the dorsal epidermis. Additional small spherical pigment granules are found scattered in the epidermis, muscle layers, and connective tissue.

Reproductive Anatomy: Externally, two male gonopores are evident, one on either side of the posterior end of the pharynx (Fig. 1B). The female gonopore is located on the midventral line between the male gonopores and the sucker. In mature animals, the uteri are triangular white masses on either side of the female gonopore; in preserved specimens they are tan colored (Fig. 1B). Ovaries are located dorsally and testes are found ventrally. Two large, oblong, and highly muscularized seminal vesicles ($925\mu\text{m} \times 600\mu\text{m}$) are present (Figs. 2A, 2E), as are two large and elongated prostatic vesicles ($550\mu\text{m} \times 350\mu\text{m}$) (Figs. 2A, 2B, 2E). Seminal and prostatic vesicles are oriented perpendicularly to each other. The prostatic and seminal vesicles are enwrapped by thick layers of circular muscles. The interiors of the prostatic vesicles are lined with columnar, glandular epithelia whose nuclei are located basally in the cells. The seminal vesicles are lined by thin ciliated epithelia. Two wide male atria house conical stylets

(350 μ m x 100 μ m) (Figs. 2B, 2E). The superficial female gonopore leads into a wide female atrium, which continues interiorly into a short and very narrow vagina. Cement pouches are well defined and surrounded by cement glands typical of pseudocerotids (Fig. 2C).

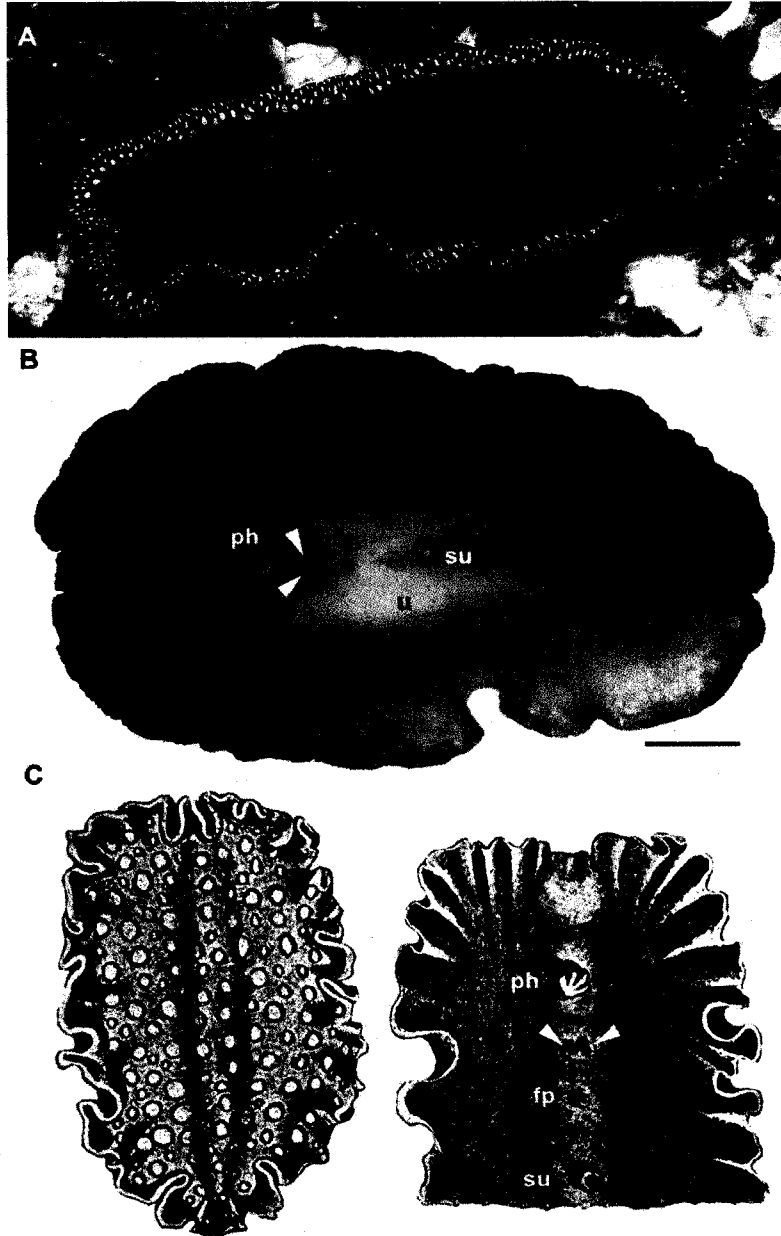


Figure 1. *Pseudobiceros pardalis* n. comb. A. Dorsal view of live animal in its natural habitat. Scale bar 5 mm. B. Ventral view of live animal showing pharynx, two male gonopores, female gonopore, uteri and sucker. Scale bar 5 mm. C. Original diagram of Verrill, 1900 of dorsal (left) and ventral view (right); ventral view clearly shows two male gonopores and pharynx, female gonopore and sucker. fp, female gonopore; ph, pharynx; su, sucker; u, uteri; arrowheads, male gonopores.

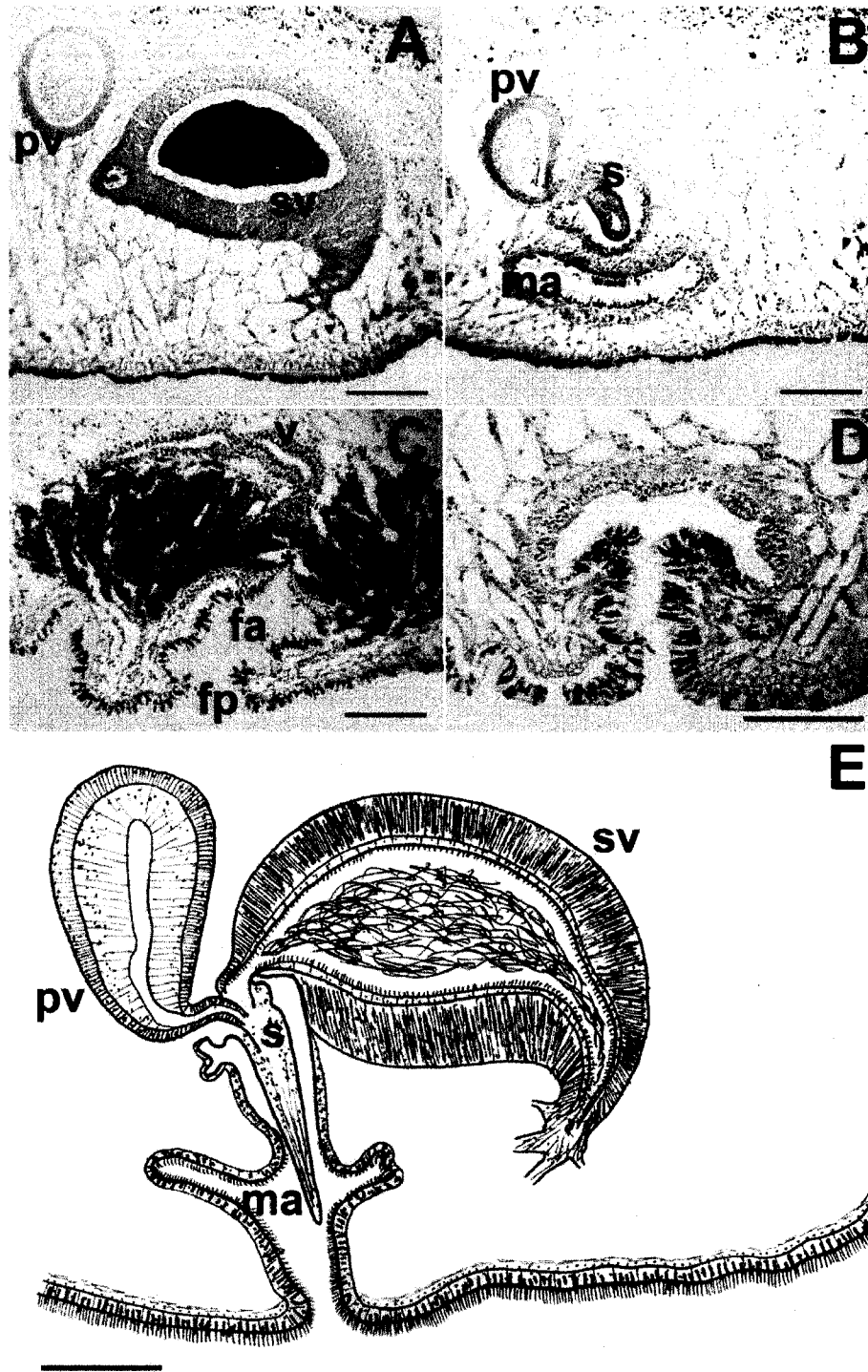


Figure 2. *Pseudobiceros pardalis* n. comb. A. Sagittal section of one male copulatory system, showing seminal and prostatic vesicles. B. Sagittal section of one male copulatory system, showing prostatic vesicle, stylet, and male atrium. C. Sagittal section of female copulatory system, showing female pore, atrium, cement glands, cement pouch and vagina. D. Sagittal section through the sucker. E. Diagrammatic representation of one male copulatory apparatus. All scale bars 250 μ m. cg, cement glands; fa, female atrium; fp, female pore; ma, male atrium; s, stylet; pv, prostatic vesicle; sv, seminal vesicle; v, vagina; asterisk, cement pouch.

Taxonomic Remarks

Pseudoceros pardalis was first described by Verrill (1900) from a single, large (60 mm x 40 mm), alcohol preserved specimen collected in Bermuda by Dr. C. H. Merriam. The description was based on external features of body shape, size and a brownish-black dorsal color pattern covered with numerous, round, pale-yellow spots (Fig. 1G). No information regarding the reproductive system was included in the original description (Verrill 1900). A second specimen, also collected in Bermuda by Dr. O. Giere in 1982, has been identified as *P. pardalis* Verrill, 1900 by Faubel (1984) and is described by having a single male copulatory apparatus.

Although our own specimens exhibit the colorations and color patterns of *Pseudoceros pardalis* Verrill, 1900, all are characterized by double male copulatory complexes. Each complex opens into an independent male gonopore, each has a seminal and a prostatic vesicle and each is armed with a penis papilla. Faubel (1984) erected the genus *Pseudobiceros* to distinguish species of *Pseudoceros* with double male copulatory systems, which allows us to establish *Pseudobiceros pardalis* n. comb. Furthermore, a closer examination of Verrill's drawing of his specimen shows the presence of two distinct male gonopores (Verrill, 1900; plate LXX, Fig. 6a) (Fig. 1G). Thus, we synonymize the single specimen of *Pseudoceros pardalis* Verrill, 1900 with *Pseudobiceros pardalis* n. comb.

It is noteworthy that the specimen of *Pseudoceros pardalis* re-described by Faubel (1984) is not the same species as the one described by Verrill (1900) despite similarities in coloration. With only one male copulatory complex, Faubel's (1984) specimen clearly belongs to *Pseudoceros*, not *Pseudobiceros*. We therefore accept *Pseudoceros pardalis* Verrill, 1900 *sensu* Faubel, 1984 as a separate, distinct species, different from *Pseudobiceros pardalis* n. comb.

***Pseudobiceros caribbensis* n. sp.**

(Figs. 3, 4)

Type Material

a) Holotype, one mature specimen (UNH-CUR 065, 17mm X 10mm) as serial sagittal sections (9 slides); USNM 1104640. Collected 21 June, 2005, subtidally under coral rubble in Curaçao, Carmabi Beach, Piscadera Baai (N12° 07.367'; W68° 68.165').

b) Paratype, one mature specimen (UNH-JAM 089, 12mm X 8mm) as serial sagittal sections (6 slides), the remaining part of the body as a whole mount (1 slide); USNM 1104686. Collected 2 June, 2006, subtidally from 6 m depth in Jamaica, St. Ann's Bay, Bull Reef (N18° 44.070'; W77° 17.355'). GenBank Accession EF514806.

Other Material Examined

c) One mature specimen (UNH-USFL 041, 20mm X 11mm) as serial sagittal sections (12 slides) USNM 1104688. Collected 2 June, 2005, subtidally from 4.5 m depth in Florida, 11-foot Mount (N24° 43.371'; W80° 51.700'). GenBank Accession EF514804.

d) One juvenile specimen (UNH-USFL 044, 16mm X 8mm) as whole mount (1 slide) USNM 1104687. Collected 2 June, 2005, subtidally from 4.5 m depth, in Florida, 11-foot Mount (N24° 43.371'; W80° 51.700'). GenBank Accession EF514805.

e) One mature specimen (UNH-CUR 069, 13mm X 7mm) as a whole mount (1 slide) USNM 1104641. Collected 23 June, 2005, subtidally in Curaçao, Playa Kalki (N12° 22.529'; W69° 09.470').

f) One immature specimen (UNH-HON 014, 4mm X 2mm) for molecular analysis only; collected 27 August, 2006, subtidally under coral rubble of a patch reef at 5 m depth in Honduras, Cayos Cochinos, Cayo Timon (N15° 56.134'; W86° 31.234').

Etymology

The name indicates the widespread distribution of this species in the Caribbean.

Synonyms

It is likely that it corresponds to *Pseudobiceros* sp. 11 Newman & Cannon (2003, p. 84), but see taxonomic remarks below.

Distribution

Known from the type locality in Curaçao. Also found in Jamaica, Florida, and Honduras. *Pseudobiceros* sp. 11 has been reported from the French Mediterranean (Newman & Cannon 2003).

Diagnosis

Background transparent brown, darker medially; raised line over the main intestine with irregular patches lacking brown pigment. Instead, numerous and densely concentrated white dots are substituting the pigment. In some cases, a fine medial dorsal white line is present. Dorsal surface covered with dark brown and white dots irregularly scattered. Larger white spots unevenly distributed around the margin.

Intestinal branches visible

through the skin as yellow net-like ramifications. Tentacle tips white with a dark brown submarginal band.

Description

External Features: Coloration as described above in diagnosis (Fig. 3A). According to the color pattern groups established for *Pseudobiceros* (Newman & Cannon 1994), the species belongs to Group 4 (spots, dots and mottling). Prominently raised medial ridge of dark brown coloration. Ventral surface brownish milkywhite with bright white scattered dots (Fig. 3B). Pointed, ear-like pseudotentacles with 87–100 eyes on each one (Figs. 3A, 3C). Cerebral eyespot horseshoe shaped with 32–34 eyes. Two additional eyes are present anterior to the brain immersed more deeply into the parenchyma than the eyes of the cerebral cluster (Fig. 3C inset). Small ruffled pharynx located anteriorly with 8–10 pharyngeal folds.

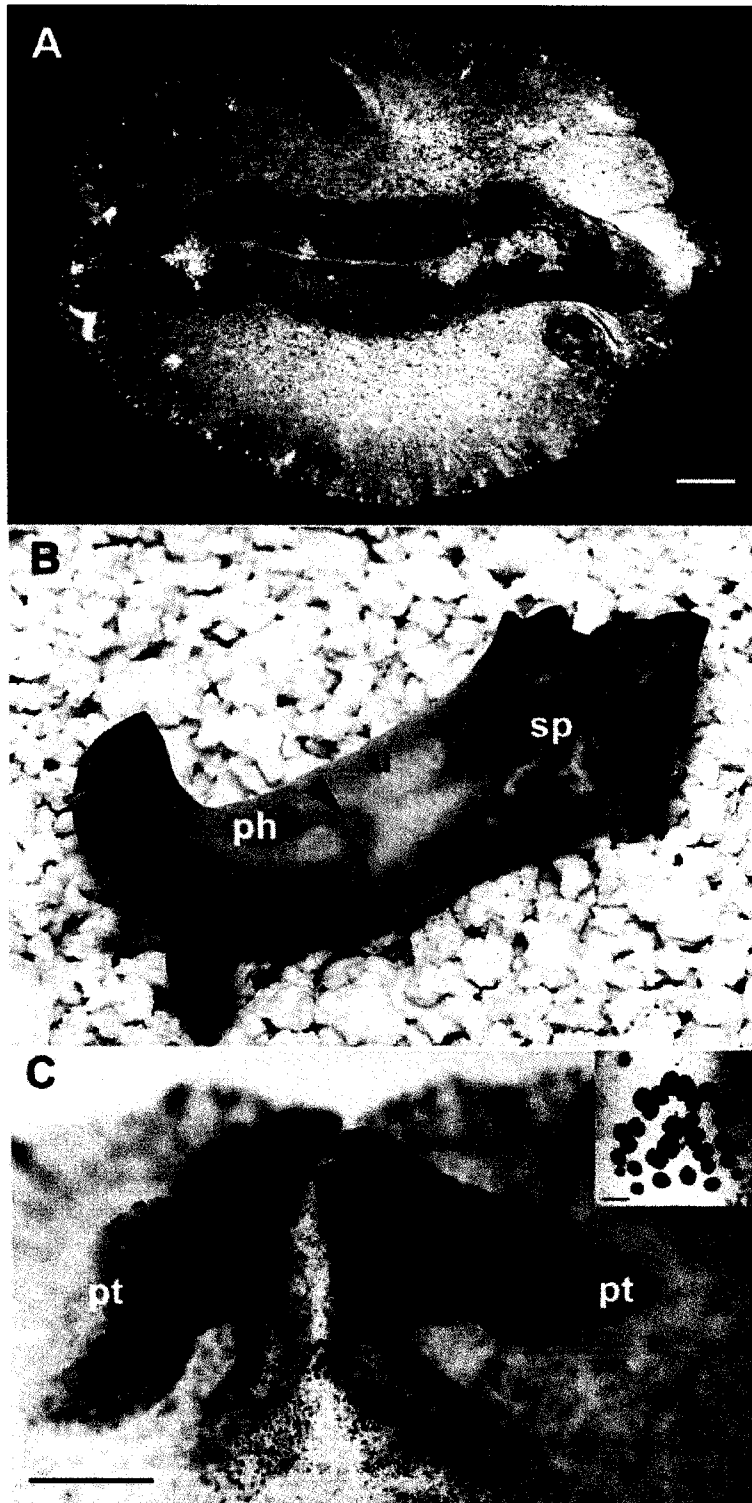


Figure 3. *Pseudobiceros caribbensis* n. sp. A. Dorsal view of live animal, showing coloration. Scale bar 1mm. B. Ventral view of live animal, showing pharynx, male gonopores, uteri, and patches of sperm from hypodermic insemination. Scale bar 5 mm. C. Cleared whole mount of the anterior end showing pseudotentacles and pseudotentacular eyes. Scale bar 0.5 mm. Inset: Higher magnification of cerebral eye cluster. Scale bar 300 μ m. ph, pharynx; pt, pseudotentacles; sp, sperm; u, uteri; arrowheads, male gonopores.

Body Wall: A simple, densely ciliated epithelium surrounds the entire body. Dorsally, the epithelial cells are somewhat taller than ventrally. Numerous glands containing slender rhabdites are found dorsally. A thin but conspicuous basement membrane is located below the epithelium. Coloration is primarily due to the contents in the intestinal branches, although small brown pigment granules are found in the dorsal epidermis and in the parenchyma. The parenchyma is coarsely granular. Individual muscle layers could not be differentiated.

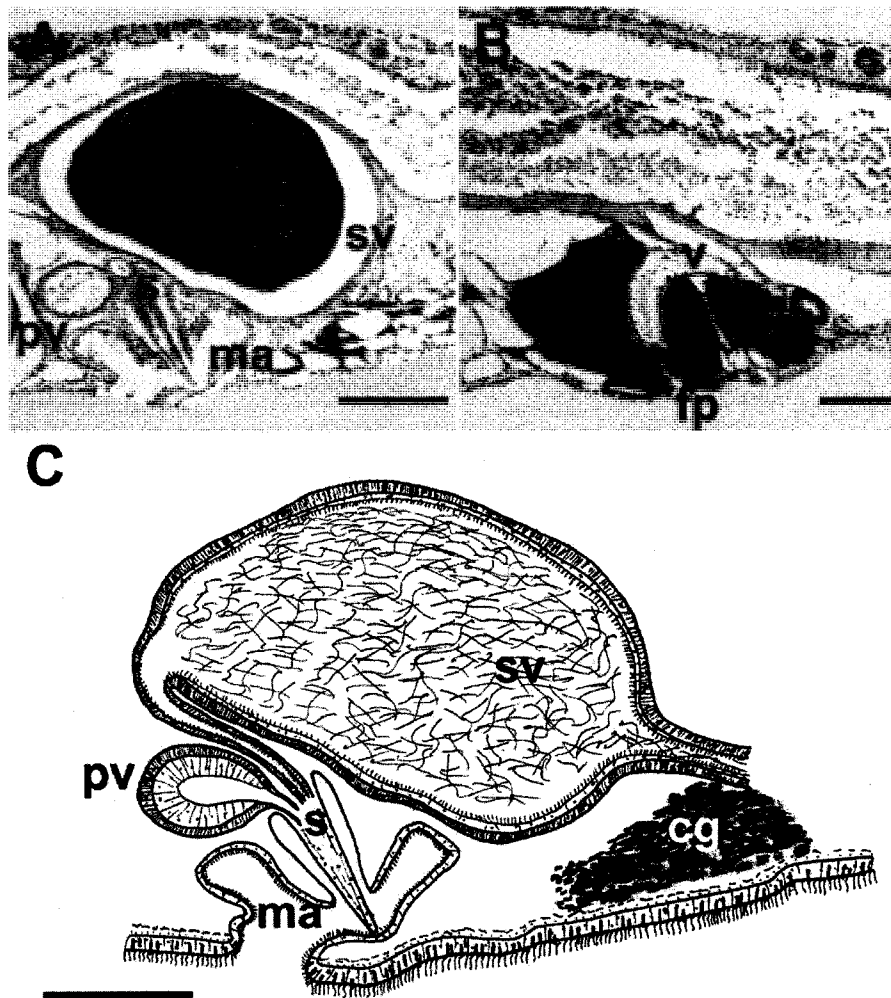


Figure 4. *Pseudobiceros caribbensis* n. sp. A. Sagittal histological sections of one male copulatory system showing seminal and prostatic vesicles, stylet, and male atrium. B. Sagittal histological section of the female copulatory system showing cement glands, vagina, and female pore. C. Diagrammatic representation of one male reproductive system. All scale bars 250 μ m. cg, cement glands; fp, female pore; ma, male atrium; s, stylet; pv, prostatic vesicle; sv, seminal vesicle; v, vagina.

Reproductive Anatomy: Externally, two separate male gonopores are evident one on either side of the posterior end of the pharynx (Fig. 3B). The midventrally located female gonopore is well separated from the male gonopores. In mature animals, the uteri appear as triangular white masses on either side of the female gonopore (Fig. 3B). Ovaries are located dorsally, testes are found ventrally. The following measurements are representative of one male copulatory system only. Measurements of the second male system are comparable.

The male reproductive system consists of a large, rounded seminal vesicle (800 μ m X 600 μ m) with a thin wall composed of circular muscles and lined by a thin ciliated epithelium. Each seminal vesicle connects to an expanded vas deferens (Figs. 4A, 4C). The expansion of the vas deferens only occurs in mature animals. The prostatic vesicle is small and narrow (300 μ m X 200 μ m) (Figs. 4A, 4C) surrounded by a thin muscular wall composed of circular fibers and lined by columnar, glandular cells that fill almost the entire lumen. There is no evidence of exterior glandular cells emptying into the prostatic vesicle. The shallow male atrium houses a long conical stylet (275 μ m). The female Atrium is small and narrow (Fig. 4B) and connects to a short and slender vagina, which is directed posteriorly. Cement glands completely surround the vagina and the small, inconspicuous cement pouches (Fig. 4B).

Taxonomic Remarks

This species was well represented in several localities in the Caribbean, and it is hard to imagine that it has not been reported previously. It is possible that due to the delicate nature of pseudocerotids and their ability to autolyse, specimens of this species had been impossible to collect and preserve in the past. Furthermore, in some specimens of *Pseudobiceros caribbensis*, the dorsal surface appears evenly dark brown in coloration, obscuring the yellow net-like ramifications of the intestinal branches and

the scattered brown and white spots. As outlined above, these characteristics are important in the determination of this new species.

The general coloration and body shape of *Pseudobiceros caribbensis* are very similar those of *Pseudoceros maximus*, a species described by Lang (1884). Lang (1884) described three mature specimens of *P. maximus*, and although they differed in their male reproductive anatomy, he considered them to be the same species. With only one male reproductive complex, the first specimen clearly belongs to the genus *Pseudoceros*. Faubel (1984) designated this as *P. maximus* (type A) and it corresponds to specimens found by Novell (2001, unpublished thesis) in the western Mediterranean. Lang's second specimen contains two complete male reproductive complexes arranged perpendicular to the long axis of the animal and opening into a single large male atrium. Faubel (1984) designated this as type B and erected the monotypic *Monobiceros langi* for it. Finally, the third specimen also is characterized by double male reproductive systems opening into a single male pore, but their arrangement is in an anterior-posterior orientation. Our specimens differ from either *Monobiceros langi* or *Pseudoceros maximus* because their double male reproductive structures open into two separate male gonopores, thus validating their placement into the genus *Pseudobiceros*. Newman & Cannon (2003) record an unidentified species of *Pseudobiceros* (sp. 11, p. 84) from the French Mediterranean, which bears a striking resemblance to *P. maximus* (type A). However, because the authors list the specimen as *Pseudobiceros*, we have to assume that it is characterized by double male reproductive systems opening into two separate male gonopores. Hence, it may be a possible synonym of *P. caribbensis*.

Final determination of this synonymy and a resolution of Lang's (1884) *P. maximus* specimens as separate species most likely will require data from nucleotide sequences.

***Pseudoceros rawlinsonae* n. sp.**

(Figs. 5, 6)

Type Material

a) Holotype, one mature specimen (UNH-USVI 029, 17mm X 7mm) as serial sections (3 slides), the remaining part of the body as a whole mount (1 slide), VIIS 41977. Collected 26 May, 2006, from coral rubble at 6 m, in the US Virgin Islands, Great Lameshur Bay, St. John (N18° 18.870'; W64° 43.361'). GenBank Accession EF514803.

Etymology

Named in honor of Dr. Kate A. Rawlinson.

Distribution

To date, found in the US Virgin Islands, Great Lameshur Bay, St. John (N18° 18.870'; W64° 43.361') and Bonaire, Netherlands Antilles (Ellen Muller, image #512, *Pseudoceros* sp. posted at <http://www.rzuser.uniheidelberg.de/~bu6/flat0512.html> and at <http://www.pbase.com/imageine/image/36592827>). To assure stable access to both web images, their URL's have been archived at <http://www.webcitation.org/5NsXWpejC> and at <http://www.webcitation.org/5NsXwnb60>, respectively via WebCite.

Diagnosis

Body white, with a black, broad, branched stripe middorsally. Margin becomes translucent towards the periphery; thin, bright orange line surrounds the entire body. Tentacles entirely black; no evidence of white body color or orange line on the tentacles. Fusiform, elongated seminal vesicle drained by an ejaculatory duct; ejaculatory duct loops dorsal over prostatic vesicle and enters penis papilla anterior to the prostatic vesicle.

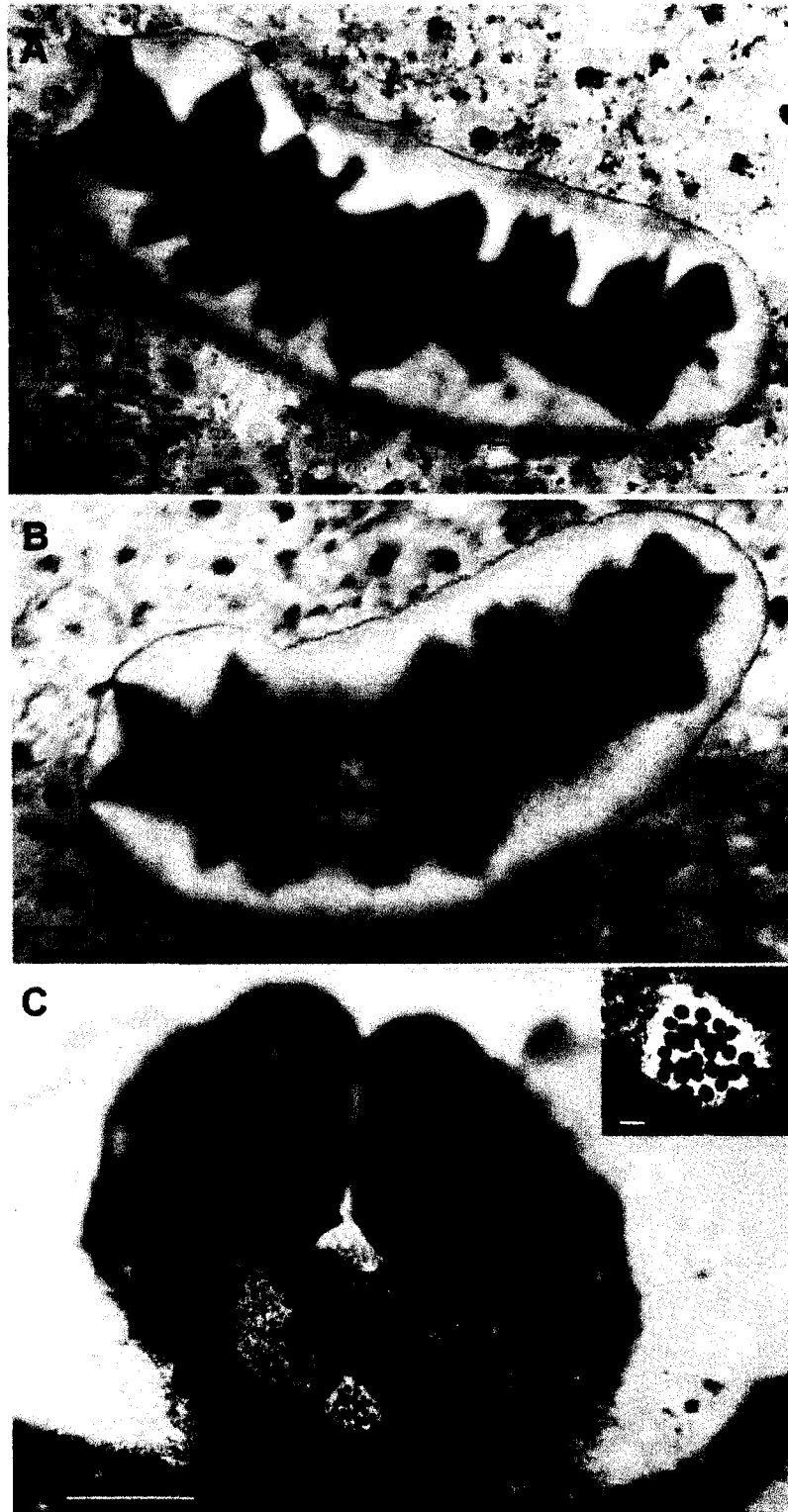


Figure 5. *Pseudoceros rawlinsonae* n. sp. A. Dorsal view of live animal in its natural habitat showing coloration and color pattern. Scale bar 2 mm. B. Ventral view of live animal, showing pharynx and uteri. Scale bar 2 mm. C. Cleared whole mount of the anterior end, showing pseudotentacles and cerebral eye cluster. Scale bar 0.5 mm. Inset: Higher magnification to show detail of cerebral eye cluster. Scale bar 500 μ m.

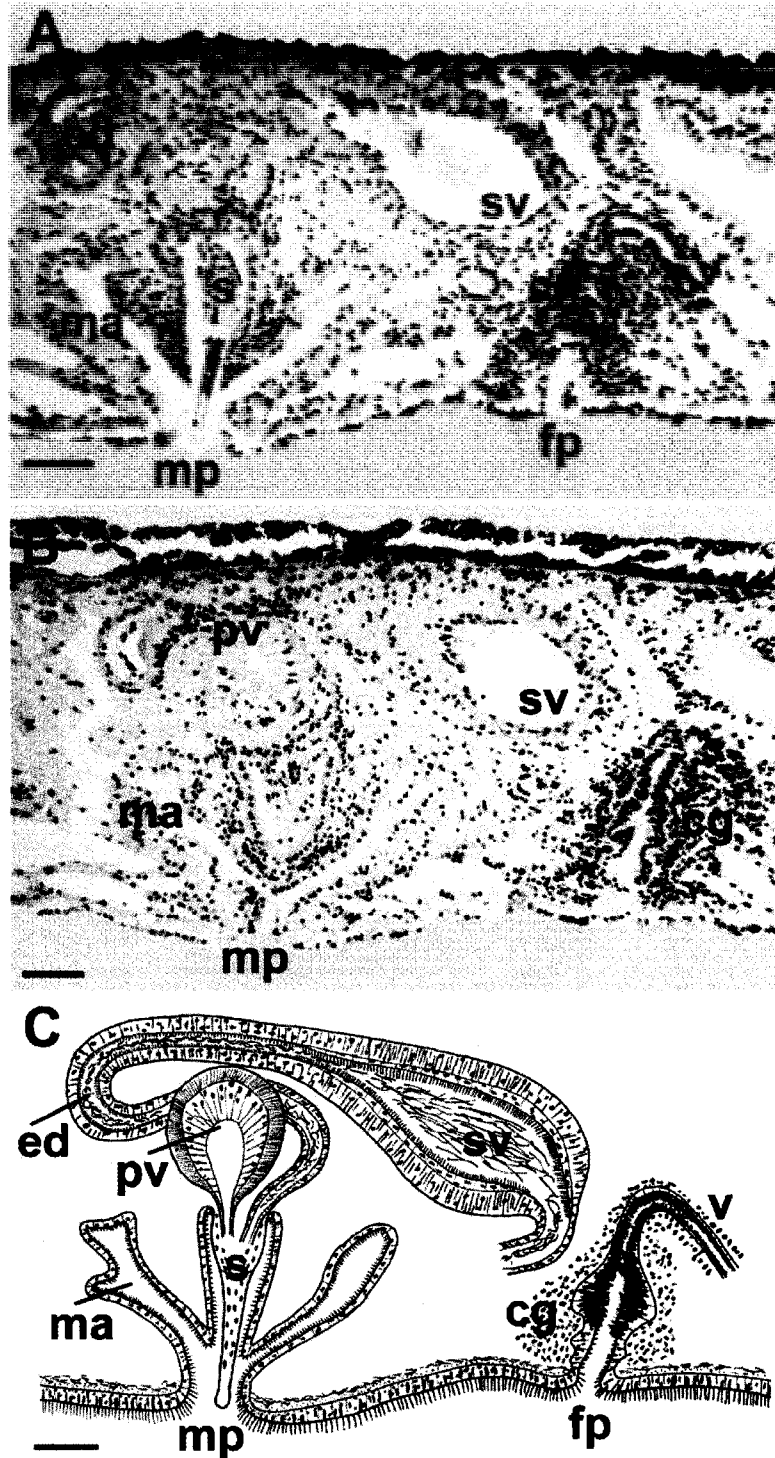


FIGURE 6. *Pseudoceros rawlinsonae* n. sp. A. Sagittal histological section of the male and female copulatory systems, showing loop of ejaculatory duct, seminal vesicle, long conical, blunt, cuticular stylet, male atrium, male and female pores, vagina, and cement glands. B. Sagittal histological section of the male and female copulatory systems, showing small, rounded prostatic vesicle, male atrium, male pore, and vagina surrounded by basophilic cement glands. C. schematic sagittal representation of the male and female reproductive systems. All scale bars 100 μ m. cg, cement glands; ed, ejaculatory duct; fp, female pore; ma, male atrium; mp, male pore; s, stylet; pv, prostatic vesicle; sv, seminal vesicle; v, vagina.

Description

External Features: Coloration as described above for diagnosis (Fig. 5A).

Following the color pattern groupings of *Pseudoceros* (Newman & Cannon 1998), this new species belongs into Group 6 (transverse streaks and stripes). Pseudotentacles are formed by simple folds of the anterior margin and are dotted with a few scattered eyes (Figs. 5A, 5C). The cerebral eyespot is rounded and contains about 36 eyes (Figs. 5C inset). The pharynx is typical of the genus. The ventral surface is translucent (Fig. 5B). In preserved animals, the distance between the male and female gonopores is 500 μm , and between the latter and the sucker it is approximately 600 μm .

Body Wall: The dorsal epidermis consists of tall, columnar cells interspersed with clusters of elongate rhabdite glands. Rhabdite glands are less numerous ventrally. The basement membrane is distinct and the typical three layers of muscles (circular, diagonal, longitudinal) can be distinguished. Dark granular pigmentation is located beneath the dorsal epidermis and among the muscle layers.

Reproductive Anatomy: The ovaries are arranged dorsally, the testes ventrally. Numerous, large and conspicuous nuclei are scattered throughout the body, and are especially concentrated in the muscle layers and the glandular areas of the reproductive systems (e. g., male atria, prostatic and ejaculatory ducts, narrowing part of prostatic vesicle). The seminal vesicle (250 μm X 550 μm) is pear-shaped, narrowing as it joins the ejaculatory duct (Figs. 6A–C). It extends antero-dorsally beyond the prostatic vesicle. The seminal vesicle is surrounded by a thick layer of circular muscles and is lined by a ciliated epithelium. The ejaculatory duct loops to enter the penis papilla anteriorly to the prostatic vesicle (Figs. 6A, 6C). The prostatic vesicle is small and rounded (175 μm) and surrounded by a thin layer of circular muscles. Its interior is lined by columnar, glandular cells, whose nuclei are located basally in the cells (Fig. 6A). The deep male atrium houses a long, conical and blunt stylet (250 μm) (Figs. 6A, 6C). The female complex has

a short and slender vagina that is surrounded by cement glands. Cement pouches are not well defined, and instead of eosinophilic cells, basophilic cells compose the cement glands, which stain not as strongly as is usual for pseudocerotids (Figs. 6A–C). The female atrium is lined by a ciliated epithelium, whereas the vagina is lined by a modified glandular epithelium.

Taxonomic Remarks

With perhaps as many as 75% of all pseudocerotid species, *Pseudoceros* represents the largest genus in the family Pseudocerotidae. Species determinations in *Pseudoceros* usually are based on coloration and color patterns (Hyman 1954, 1955a, b, 1959a, b; Prudhoe 1989; Newman & Cannon 1994, 1996, 1997, 1998) because of a lack of distinguishing characters in their reproductive anatomies. The newly described species

Pseudoceros rawlinsonae exhibits a unique, and as of yet, undescribed color pattern (Fig. 6A). Other species exhibiting similar color patterns with a median, branched stripe include *Pseudoceros imperatus* and *P. zebra*. However, the body of *P. imperatus* is very dark brown to almost black, the middorsal stripe is yellow-green with only a few wide branches, and the marginal band is very broad, orange, and extends across the tentacles (Newman & Cannon 1998). In *P. zebra*, the body is black, the stripe is white with wide branches and the marginal band is yellow, very wide and extends across the tips of the tentacles.

***Pseudoceros harrisi* n. sp.**

(Fig. 7)

Type Material

a) Holotype, one mature specimen (UNH-PAN 036, 11mm X 7mm) as serial sections (4 slides) USNM1104642. Collected 19 June, 2005, from coral rubble at 4.5 m depth by Dr. Larry Harris in Panama, Bocas del Toro, Isla de los Pájaros (N9° 27.156'; W28° 19.975'). GenBank Accession EF514802.

Etymology

Named after the collector, Dr. Larry Harris.

Distribution

To date, known only from the type locality.

Diagnosis

Transparent cream-pinkish background. Middle area of dorsal surface with a strong red coloration but absent towards the margin. Big white spots over the red pigment. Smaller and numerous white dots forming a conspicuous margin. Bright white tentacles. According to the coloration pattern groupings of Newman & Cannon (1998), this species fits into Group 5.

Description

External Features: Coloration as described above for diagnosis (Fig. 7A). The bright white pseudotentacles are formed by simple folds of the anterior margin. The cerebral eyespots are small (Fig. 7A). The pharynx is typical for the genus. Because only one specimen was found, the entire animal was sectioned. The distances given below were taken from the histological slides.

Body Wall: The entire animal is surrounded by a ciliated, columnar epithelium with interspersed rhabdite glands. No pigment granules are evident.

Reproductive Anatomy: The ovaries are located dorsally in the animal, the testes are ventral. The seminal vesicle (350µm X 225µm) is oval, with a strongly muscularized wall and a ciliated epithelium lining the lumen. A smaller, rounded prostatic vesicle (150µm X 112µm) is located ventral to the seminal vesicle and is surrounded by a thin

layer of circular muscles and lined by tall, columnar glandular cells. A deep male atrium houses a long stylet (125 μ m) that is directed forwards (Figs. 7B–C). The female reproductive system has a long and slender vagina and well-defined cement pouches. Cement glands surround the proximal part of the vagina (Figs. 7B–C). The vagina is lined by a ciliated epithelium. The distance between gonopores is 500 μ m and from the female gonopore to the sucker 900 μ m.

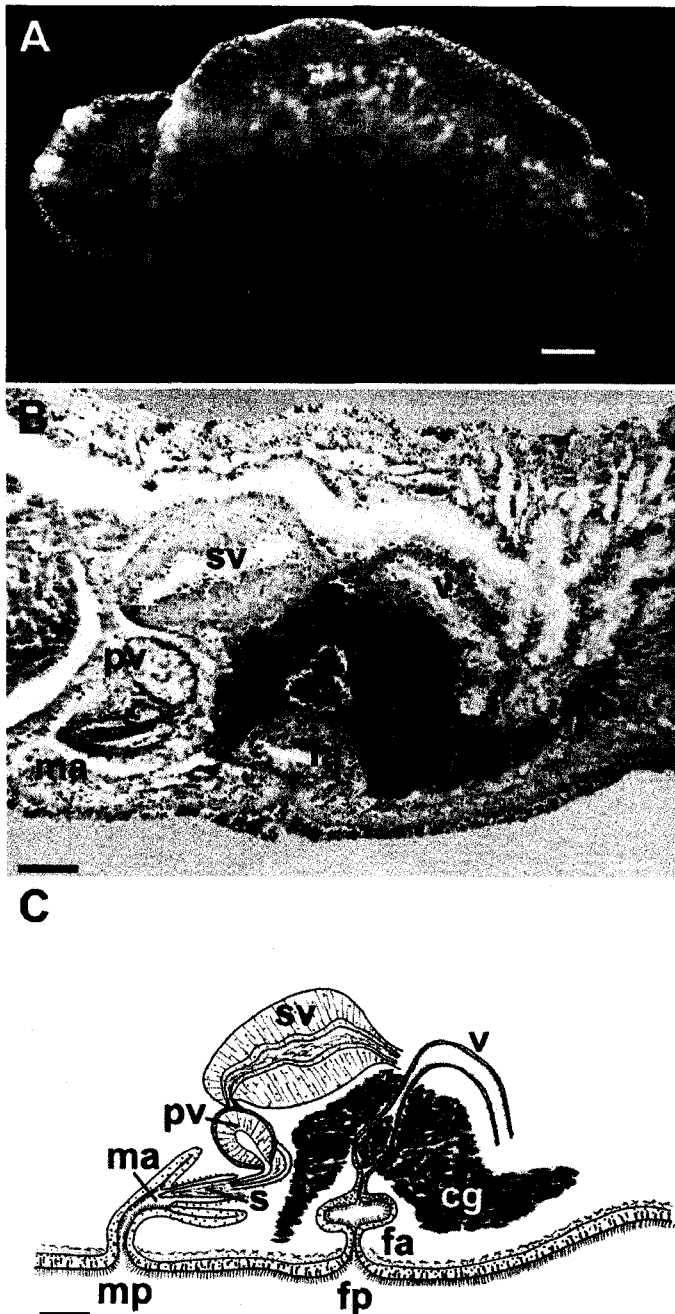


Figure 7. *Pseudoceros harrisi* n. sp. A. Dorsal view of live animal, showing tentacles and cerebral eye cluster (anterior end broken). B. Sagittal histological section of male and female copulatory systems, showing seminal and prostatic vesicles, stylet, male and female atria, cement glands, and vagina. C. Diagrammatic representation of male and female reproductive systems. All scale bars 100 μ m. cg, cement glands; fa, female atrium; fp, female pore; ma, male atrium; mp, male pore; s, stylet; pv, prostatic vesicle; sv, seminal vesicle; v, vagina.

Taxonomic Remarks

Unfortunately only one damaged specimen was found and therefore, comparisons of color variation within the species were not possible. This species does not resemble any other species of *Pseudoceros*. However, superficially *P. harrisi* may be confused with members of *Cycloporus* because of its body shape, the mottled coloration and the marginal dots around the body. But with a more carefully examination, *P. harrisi* can be distinguished easily from *Cycloporus* because these marginal dots are due to pigmentation only and not to the peripheral vesicles that open to the exterior as found in *Cycloporus*. Additionally, the pharynx is ruffled, only one cluster of cerebral eyes is present, and the pseudotentacles are well defined.

***Thysanozoon raphaeli* n. sp.**

(Fig. 8)

Type Material

- a) Holotype, one mature specimen (UNH-BLZ 057, 15mm X 10mm) as serial sagittal sections (8 slides), remaining part of the body as a whole mount (1 slide) USNM 1104643. Collected among mangroves in Belize, Twin Caye (N16° 49.46', W88° 06.068') in June 2006 by Raphael Ritson-Williams. GenBank Accession EF514810.
- b) Paratype, one juvenile specimen (UNH-PAN 001, 8mm X 4mm) as whole mount; collected 12 June, 2005, among mangroves (0.5 m depth) in Panama, Bocas del Toro, Sunset Point (N9° 17.829'; W82° 15.848'). USNM 1104689.
- c) Paratype, one juvenile specimen (UNH-PAN 049, 3.5mm X 3.0mm) as whole mount (1 slide) USNM1104644. Collected 20 June, 2005, from the water column at 1.2 m depth in Panama, Bocas del Toro, Punta Coco (N9° 17.829'; W82° 15.848'). GenBank Accession EF514809.

Other Material Examined

d) One juvenile specimen (UNH-PAN 050, 6mm X 3mm) as whole mount; collected subtidally from 4.5 m depth on 21 June, 2005, in Panama, Bocas del Toro, Crawl Cay (N9° 14.563'; W82° 08.302').

Etymology

Named for Raphael Ritson-Williams who provided us with the type specimen.

Synonyms

It is possible that this species corresponds to *Thysanozoon* or *Acanthozoon* sp. 4 Newman & Cannon 2003 (p. 86) because of similarities in body shape, papillae, and coloration.

Distribution

This species was found in Belize, Panama and Colombia.

Diagnosis

Translucent brown-blackish background with numerous large bulbous yellow papillae over the entire dorsal surface. Small white slash-like marks, hardly visible, around the entire margin, sometimes giving the impression of an extremely thin white border. Darker pigment medially and darker black tentacles outlined by the white marks. Translucent white ventrally.

Description

External Features: Coloration as described above for the diagnosis (Figs. 8A–C). Short, bulbous papillae are dispersed over the dorsal surface; with long and pointed pseudotentacles (Figs. 8A–B). One oval cerebral eyespot is present (Fig. 8C inset), although because of the dark background pigment, the number of eyes in the cluster and on the pseudotentacles could not be determined.

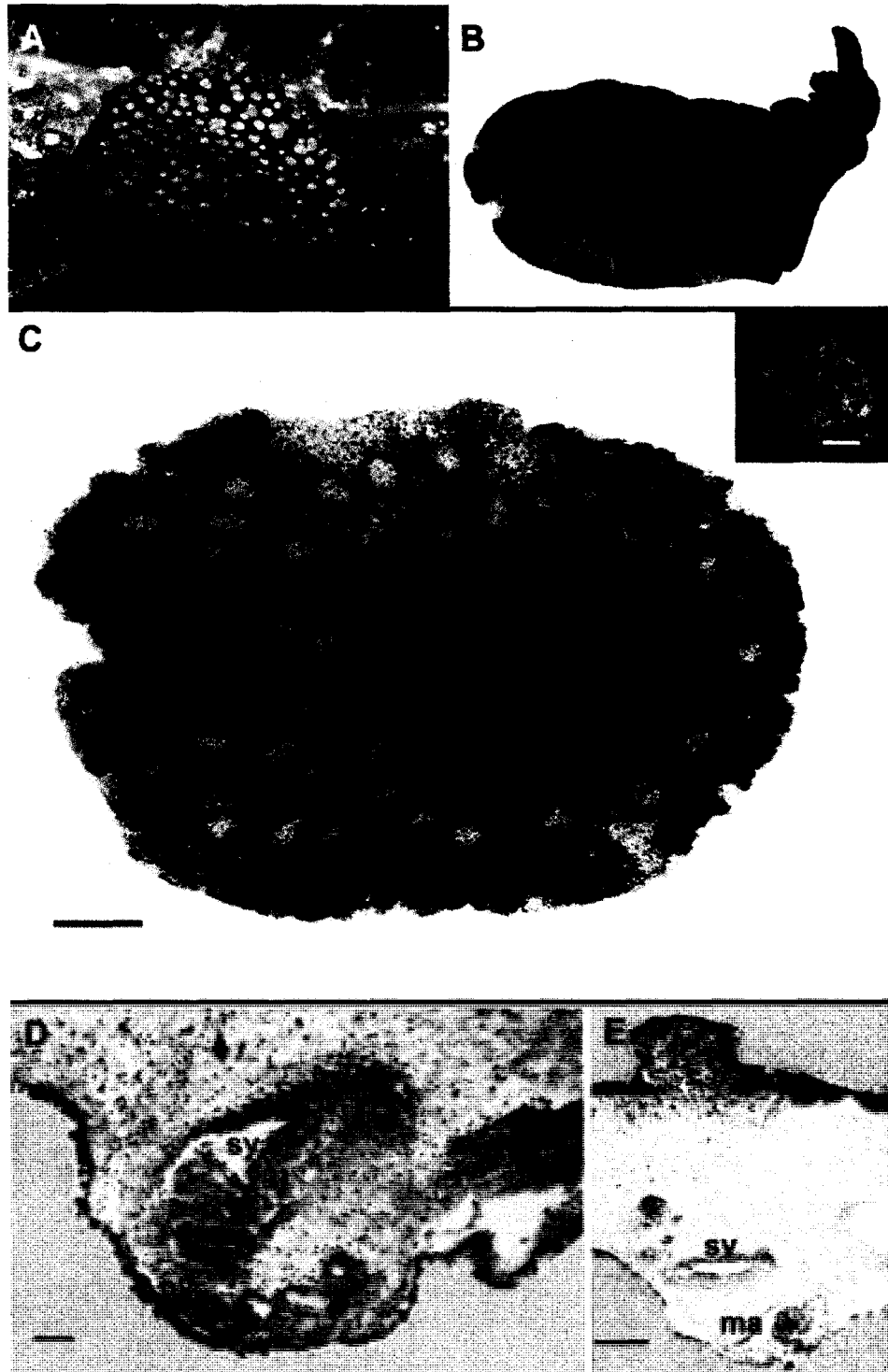


Figure 8. *Thysanozoon raphaeli* n. sp. A. Dorsal view of live animal in mangrove roots at Twin Caye, Belize. Photo credit, Raphael Ritson-Williams. B. Dorsal view of live animal found in Bocas del Toro, Panama. Photo credit, Dr. Arthur Anker. C. Dorsal view of cleared whole mount. Scale bar 1.5 mm. Inset: Higher magnification to show detail of cerebral eye cluster. Scale bar 500 µm. D. Sagittal histological section of one male copulatory system, showing seminal and prostatic vesicles. Scale bar 100 µm. E. Sagittal histological section of one male copulatory system, showing seminal vesicle and male atrium. Scale bar 200 µm. ma, male atrium; pv, prostatic vesicle; sv, seminal vesicle.

Body Wall: A ciliated, columnar epithelium surrounds the entire specimen. In addition to rhabdite glands, a second type of glands is very common. The secretions within this second type are strongly acidophilic. These types of glands are conspicuous in the ventral epidermis and in the epithelium covering the papillae (Fig. 8E).

Reproductive Anatomy: All information is from only one specimen because the other collected specimens were immature juveniles. The seminal vesicle is large, oval in shape (450 μ m X 225 μ m) and close to the body wall (Figs. 8D–E). The small, oval prostatic vesicle (100 μ m X 75 μ m) is located ventrally (Fig. 8D). The male atrium is not well defined in the sections but a stylet is discernible (Fig. 8E). It was not possible to visualize the internal female reproductive system. The distance between the female gonopore and the sucker is 1200 μ m.

Taxonomic Remarks

In some juvenile specimens of *Thysanozoon raphaeli*, the dorsal papillae are less numerous but still about the same size as in the adults. We noted the shape and distribution of the papillae in *T. raphaeli* are similar to those of *Thysanozoon* or *Acanthozoon* sp. 4 (Newman & Cannon 2003, p. 86), although the color of the papillae in that species is white instead of yellow. Additional similarities include body shape and background coloration. Thus, it is possible that their undescribed species is *T. raphaeli*. The genera *Thysanozoon* and *Acanthozoon* are distinguished by two vs. one male gonopores, respectively. However, because Newman & Cannon (2003) did not indicate the number of male gonopores for their specimen, we cannot be absolutely certain that it is the same species as *T. raphaeli*, and therefore, will leave it as a possible synonym only.

Superfamily: Euryleptoidea Lang, 1884

Family: Euryleptidae Faubel, 1984

***Maritigrella newmana* n. sp.**

(Figs. 9, 10)

Type Material

a) Holotype, one mature specimen (UNH-USVI 0100, 18mm X 7mm) as serial sagittal sections (4 slides), the remaining part of the body as a whole mount (1 slide) VIIS 41978. Collected 3 June, 2006, from the intertidal in coral rubble in the US Virgin Islands, Salt Pond Bay, St. John (N18° 18.454'; W64° 43.440'). GenBank Accession EF514801.

b) Paratype, one mature specimen (UNH-USVI 034, 20mm X 12mm) as a whole mount (1 slide) VIIS 41979. Collected 26 May, 2006, from coral rubble at 4.5 m depth in the US Virgin Islands, Great Lameshur Bay, St. John (N18° 18.870'; W64° 43.36'). GenBank Accession EF514800.

c) Paratype, one juvenile specimen (UNH-BLZ 032, 9mm X 4mm) as serial sagittal sections (4 slides), the remaining part of the body as a whole mount (1 slide) USNM 1104645. Collected 22 June, 2006, intertidally from coral rubble in Belize, Southwater Caye, North End (N16° 49.113'; W88° 04.818'). GenBank Accession EF514798.

Other Material Examined

d) One juvenile specimen (UNH-BLZ 040, 8mm X 3mm) preserved in ethanol; collected 22 June, 2006, intertidally from coral rubble in Belize, Southwater Caye, North End (N16° 49.113'; W88° 04.818'). GenBank Accession EF514799.

e) One mature specimen (UNH-HON 036, 15mm X 6mm) as serial sagittal sections, the remaining part of the body as a whole mount; collected 3 September, 2006, at 1.5 m from coral rubble in Honduras, Cayos Cochinos, Cayo Menor (N15° 57.793'; W86° 30.727').

f) One juvenile specimen (UNH-HON 004, 10mm X 4mm) as a whole mount; collected 25 August, 2006 from coral rubble, in Honduras, Cayos Cochinos, Pelicano 3 (N15° 58.431'; W86° 28.436').

Etymology

The species is named in honor of Dr. Leslie Newman who described the genus *Maritigrella*.

Synonyms

It is likely that it corresponds to euryleptid sp. 11 Newman & Cannon (2003, p. 67) based on coloration and color pattern.

Distribution

Common species, found at the type locality in the US Virgin Islands, and also found in Belize, Honduras, Colombia, and probably Dominica (Newman & Cannon 2003, p. 67).

Diagnosis

White background with brown pigment arranged in dense, anastomosing, scale-like outlines. Dense brown pigment covering entire dorsal surface but absent near the margins; white median area without pigment visible over the pharynx and extending posteriorly. Three to five conspicuous and continuous transverse black stripes, well-spaced across the dorsal surface (may vary with the size of the animal); series of finer, grayishblack, transverse stripes located between the five conspicuous transverse stripes and extending from the margins towards the midline of the animal but do not completely cross the dorsal surface. Narrow, orange, marginal line surrounding the body but anteriorly crossing over the dorsal side behind the tentacles. Black tentacles with white tips and white coloration of the anterior margin between the tentacles. Ventral side is translucent white.

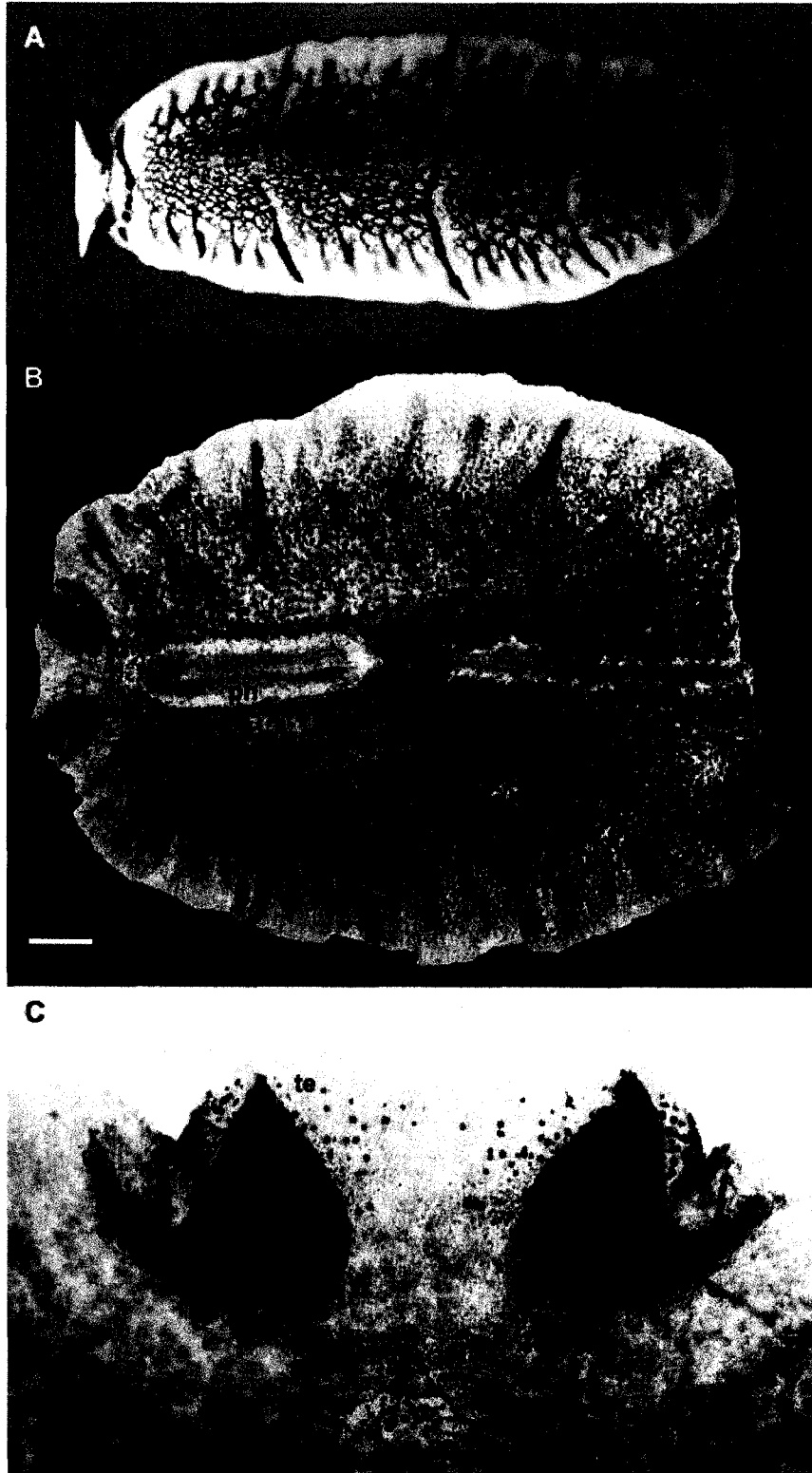


Figure 9. *Maritigrella newmana* n. sp. A. Dorsal view of live animal, showing coloration and color pattern. Scale bar 2.5 mm. B. Cleared whole mount of animal showing tentacles, cerebral eye cluster, pharynx and uteri. Scale bar 1 mm. C. Cleared whole mount of the anterior end showing details of tentacles, cerebral eye cluster and tentacular eyes. Scale bar 0.5 mm. ce, cerebral eyes; ph, pharynx; te, tentacular eyes, u, uteri.

Description

External Features: Coloration as described above in diagnosis (Fig. 9A). Pointed tentacles that are held erect (Figs. 9A–C), with a few scattered eyes between them (Figs. 9B–C). There are two cerebral eye clusters with about 26 eyes in each one (Fig. 9C). The pharynx (3.6mm X 1mm) is muscular and tubular (Fig. 9B). The paired uteri have no uterine glands. The distance between the gonopores is 400µm and 1200µm between the female gonopore and the sucker. The sucker is large and conspicuous (325µm in diameter).

Body Wall: A simple, ciliated epithelium surrounds the entire animal. There is no difference in the height of the cells between the dorsal and the ventral epidermis, although rhabdite glands are more abundant dorsally. Thick, distinct basement membrane separates the epithelium from the underlying muscle layers (outer circular, middle diagonal, inner longitudinal). Small clusters of cyanophilous pigment are located immediately below the dorsal epidermis.

Reproductive Anatomy: The ovaries are located dorsally, the testes are scattered throughout the body ventrally. A large, irregularly shaped seminal vesicle (550µm X 200µm) with thickly muscularized walls is located adjacent to one side of the prostatic vesicle (Figs. 10A, 10C). Its lumen is lined by a ciliated epithelium. The prostatic vesicle is large and oval (400µm X 300µm) (Figs. 10B–C), surrounded by a thin layer of circular muscles and lined by a glandular epithelium that consists of tall cells and whose nuclei are located peripherally. Additionally, the prostatic vesicle is surrounded by glandular cells whose thin necks appear to penetrate the prostatic wall (Fig. 10B). The penis papilla is well developed and bears a short stylet (150µm) (Figs. 10B–C). In our sectioned specimen, the vasa deferentia are expanded and filled with sperm. The female reproductive system is characterized by a deep vagina and well-developed cement pouches. The epithelium of the cement pouches is glandular. The female atrium and the

vagina is lined by a ciliated epithelium. Strong cement glands extend dorsally along the side of the vagina (Figs. 10A, 10C).

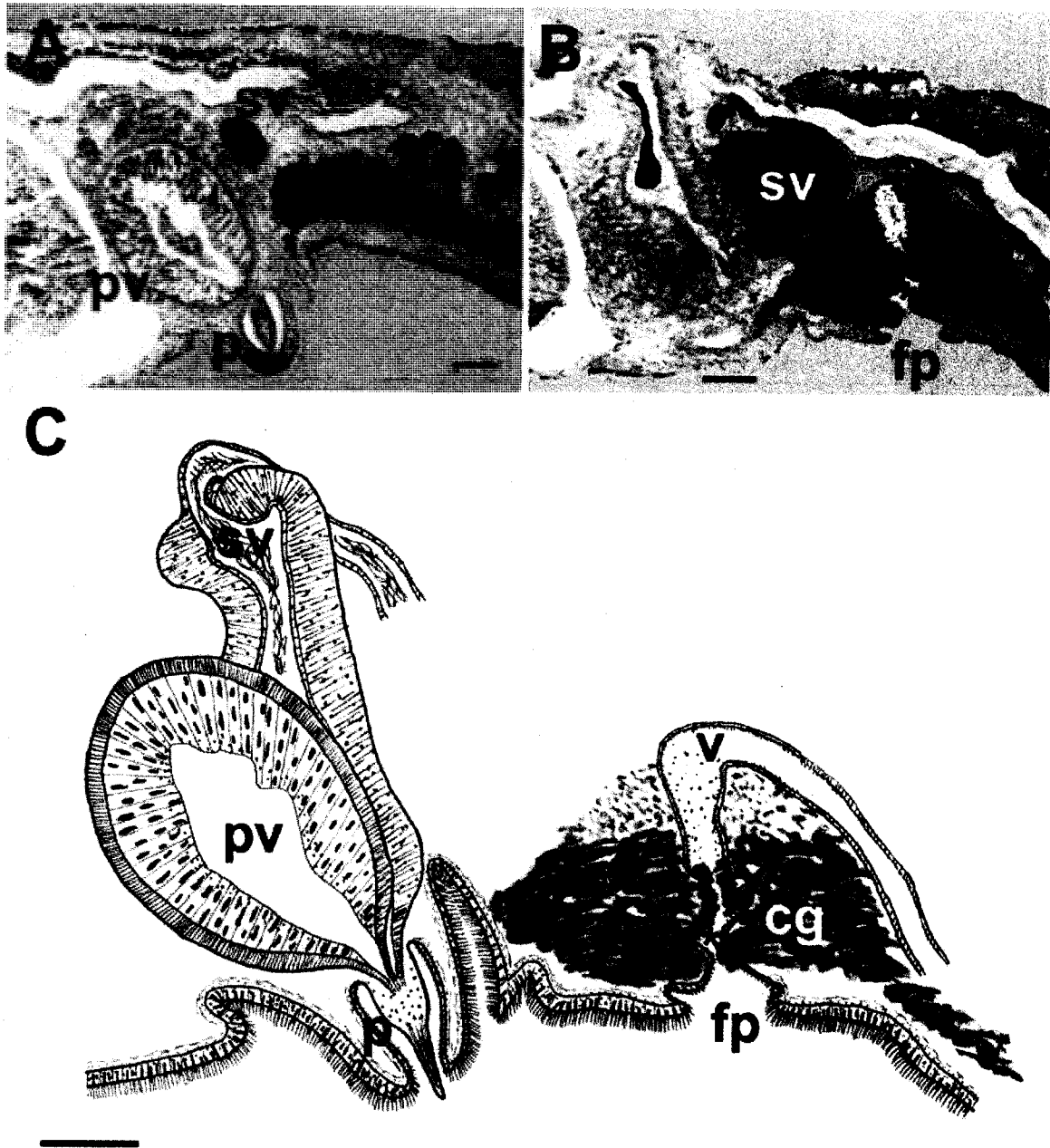


Figure 10. *Maritigrella newmana* n. sp. A. Sagittal histological section of male copulatory system, showing large, oval prostatic vesicle and penis papilla. B. Sagittal histological section of male and female copulatory systems, showing large, amorphous seminal vesicle, deep vagina, cement pouch, and well-developed cement glands. C. Diagrammatic representation of male and female reproductive systems. All scale bars 100 μ m. cg, cement glands; fp, female pore; p, penis papilla; pv, prostatic vesicle; sv, seminal vesicle; v, vagina; asterisks, cement pouch.

Taxonomic Remarks

This species clearly belongs to *Maritigrella*. It exhibits the typical characteristics of the genus, such as an oval body shape, a small, muscular and tubular pharynx, long tentacles that are held erect, a short, pointed, and sclerotized stylet a lack of uterine vesicles, and especially, the distinct striped color pattern for which the genus is known (Newman & Cannon 2000). *M. newmanae* can be differentiated clearly from other species in the genus by the distinct brown pigment pattern on its dorsal surface, the discrete black transverse stripes extending completely across the dorsal surface, and the marginal orange line around the body. In juveniles, the dorsal pigment may take on a more reddish tint instead of being dark brown. The transverse stripes vary in number depending on the size of the animal, and in some cases, they can be interrupted at the midline. In some specimens, the transverse stripes are absent over the area of the brown pigment. In those cases, the stripes are limited to the margin only, with just their beginnings being visible.

Other species of *Maritigrella* that exhibit brown pigment patterns middorsally and black transverse stripes or lines include *M. fuscopunctata*, *M. marygarsonae*, and *M. virgulata* (Newman & Cannon 2000). *M. newmanae* is distinguished from *M. fuscopunctata* by the fact that the middorsal area of *M. fuscopunctata* contains orange-brown solid spots arranged in a honeycomb pattern and none of the marginal transverse black lines extend across the dorsal surface. In *M. marygarsonae*, the brown pigment is confined to a thin middorsal line and the black transverse markings consist of numerous, thin lines that extend from the margin to the middorsal line. Finally, our species is distinct from *M. virgulata* by its scale-like brown network middorsally, and by black transverse streaks that cross over the entire dorsal surface (Newman & Cannon 2000). Furthermore, none of the other describes species of *Maritigrella* have an orange marginal line.

Discussion

Some of the specimens were found associated with their prey items. Most *Maritigrella newmanae* from either the US Virgin Islands or Belize were found consistently within transparent ascidians, probably feeding on them but possibly also seeking refuge from their own predators. Some specimens of *Thysanozoon raphaeli* were found feeding on the fouling communities associated with mangrove roots. No prey items were found with *Pseudoceros rawlinsonae*, *P. harrisi*, or *Pseudobiceros caribbensis*.

Polyclad flatworms are almost exclusively marine and have been recorded from tropical, temperate and even arctic waters (Prudhoe 1989). They also are known from the deep-sea (Quiroga et al. 2006). Due to a focused and long-term sampling effort, some of the highest diversity of polyclads (more than 600 species) has been recorded from the Great Barrier Reef and the Indo-Pacific (see extensive references in Newman & Cannon 2003). In contrast, polyclad diversity in the Caribbean has received much less attention (Bolaños et al. 2006, Hyman 1939a, b 1955a, c, Marcus 1960, Marcus & Marcus 1968, Prudhoe 1944, Quiroga et al. 2004).

The present study adds five new cotyleans to the known polyclad diversity of the Caribbean, increasing the total number to 128 species. The new species descriptions presented here are the result of a survey of eight Caribbean localities over two field seasons and may be an indication that pseudocerotid and euryleptid flatworms are more common in the tropical waters of the Caribbean than anticipated from earlier reports (Hyman 1939a, b 1955a, c, Marcus 1960, Marcus & Marcus 1968, Prudhoe 1944). However, in comparison with the Indo-Pacific, cotylean diversity in the Caribbean is relatively low. This discrepancy may be explained in part by the deterioration of many Caribbean coral reefs, the difficulty in collecting these fragile animals, their cryptic nature, and until now, a lack of focused surveys. Furthermore, only a few Caribbean

cotyleans exhibit the flamboyant coloration and color patterns so characteristic of species from the tropical waters of the Indo-Pacific, making them more difficult to detect. An on-going survey in the Caribbean combined with improved methods for collecting and preserving these delicate flatworms will hopefully help in elucidating their biodiversity and distribution.

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APPENDIX C

FIXATION AND EMBEDDING PROTOCOL FOR HISTOLOGY

Fixation and Preservation: Using a fine paint brush, transfer the specimen onto a wet piece of filter paper and place the filter paper on a frozen block of 10% buffer-formalin. Add a thin layer of cold fixative or seawater and use a paint brush to keep the animal flat as it is fixed. Fix at room temperature or 4°C for at least 24 h (duration of fixation depends on the size of the specimen). After fixation, rinse the specimens twice with 70% ethanol for 15 min each. If specimens are not processed for histology immediately, samples can be stored in 70% ethanol for an extended period of time.

Dehydration: Decant the 70% ETOH and add 95% ETOH for 30 m, process specimens through two changes of 100% ETOH for 30 min each. Combine 100% ethanol and HistoClear (50/50) and place tissue into mixture for 30 min. Clear with 100% HistoClear for 30 min (have the melted paraffin ready for the next step).

Embedding: Transfer the tissue to a mixture of HistoClear and paraffin (50/50) for 45 min at 56°C. Transfer to a paraffin/HistoClear (70/30) for 45 min and finish with pure paraffin for 45 min at 56°C. Place tissue into a second change of paraffin and leave it for at least 12 h (to assure more complete penetration of the paraffin into the tissues samples, leave overnight in paraffin). Transfer to a third change of paraffin for 1 h at 56°C. Clean metal mold and heat at 56°C for 5 minutes. Fill the mold with liquid paraffin, and adjust the tissue in the desire orientation (sagittal, longitudinal, etc). Place a labeled block stub on top of the mold and gently add more paraffin. Wait about 5 minutes for the paraffin to

solidify and then place the block in the refrigerator for 2 h. If immediate sectioning is planned, allow the block to warm to room temperature for about 20 min. Blocks can be stored for several months at 4 °C. Trim and cut the block. Collect sections of about 5-7 µm on poly-L lysine coated or charged glass slides. Proceed with staining.

Staining with Hematoxylin and Eosin (H&E):

Hydration:	Histoclear	1X	10min
	Histoclear	1X	5 min
	100% ETOH	2X	5 min
	95% ETOH	1X	3 min
	70 % ETOH	1X	3 min
	50% ETOH	1X	3 min
	Distilled water		5 min
Stain:	Hematoxylin		3 min
	Rinse with tap water		
	Acidic ethanol solution		immerse 8-10 times (to remove background)
	Rinse with tap water		
	Ammonia water		immerse 4-6 times (to intensify blue staining)
	Rinse with tap water		
	Eosin		35 sec

Dehydration: 50% ETOH	1X	3 min
70% ETOH	1X	3 min
95 % ETOH	1X	3 min
100% ETOH	2X	5 min
Histoclear	2X	5 min

Air dry slides overnight and then mount them by placing a few drops of Permount on the sections and cover with a cover slip. After a week when slides are completely dry, seal them with transparent nail polish.

Acidic ethanol solution: 0.25 ml of HCl
100 ml of 70% ethanol

Ammonia water: 99 ml of dH₂O
1 ml of Ammonium Hydroxide

Preparation of whole mounts: Using the entire worm or the remaining parts, follow the procedure for dehydration after fixation. After clearing the samples with Histoclear mount the samples in Permount on a glass slide and cover with a cover slip. Allow to dry for several days.

APPENDIX D

FIXATION AND STAINING PROTOCOL FOR PHALLODIN

Fixation and staining: Fixed the animal in 4% paraformaldehyde for 2 h at room temperature. Rinse for 5 minutes with phosphate-buffered saline (PBS, pH 7.4), permeabilize with 0.2 Triton X-100 in PBS for 1 h at room temperature, and stain the animal with Alexa-Fluor 488-conjugated phalloidin overnight at room temperature in the dark. Cover with a Petri dish to prevent evaporation. Rinse the specimen 3 times in PBS for 5 minutes each, and mount the sample with Gel Mount on a 22x40 slide, add an additional drop of GelMount if necessary and add 18x18 coverslip. Store in the dark in a slide box at 4°C or -20°C.

Preparation of Solutions:

a) 4% Paraformaldehyde in PBS

1. Mix 0.8 g paraformaldehyde in 10 ml deionized water at 60 °C.
2. Add XX how many ?drops of NaOH (Stir)
3. Add 2 ml 10X PBS
4. Bring volume to 20 ml with deionized water.

b) Phosphate-buffered Saline (PBS)

NaCl	8g
KCl	0.2g
K-phosphate monobasic	0.2g
Na-phosphate dibasic	1.14g

1. Stock solution: Add above to 80 ml distilled water and adjust pH to 7.4, then fill to 100 ml (PBS 0.1M).

2. PBS working solution (1:10): For PBS 0.01M, dilute 1 ml PBS stock solution with 10 ml distilled water.

c) 0.2% Triton X-100 in PBS

200 μ l Triton + 100 ml PBS

d) Alexa-Fluor 488-conjugated Phalloidin

Stock solution: To 300 units of Alexa Fluor 488 Phalloidin (Molecular Probes, Oregon) add 1.5 ml of methanol to dissolve the phalloidin. Final concentration 200 units/ml.

Working solution: Use 10 μ l of stock solution and place into a 500 μ l microfuge tube.

Let methanol evaporate completely in the dark for 30 minutes.