RESEARCH NOTES

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Germinal Elements and Their Development in *Echinostoma caproni* and *Echinostoma paraensei* (Trematoda) Miracidia

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ABSTRACT: Among the large cells located in the posterior of *Echinostoma caproni* and *E. paraensei* miracidia are secretory cells, germinal cells (GC), and undifferentiated cells. Secretory cells do not give rise to progeny, whereas GC do. Undifferentiated cells develop into GC that can also divide to produce embryos. Cleavage of GC of *E. caproni* occurs only after the parasite has entered the snail host and develops into a sporocyst. With *E. paraensei,* GC are larger than noted for *E. caproni,* and in 3 of 23 miracidia examined, germinal cell cleavage had occurred in the miracidium such that an embryo containing 20–25 blastomeres was present. Observations on the germinal elements of miracidia help to explain previous results showing that (1) *E. paraensei* sporocysts release mother rediae a few days earlier than do sporocysts of *E. caproni,* and that (2) a single mother redia is produced ahead of all others by sporocysts of *E. paraensei,* but not by sporocysts of *E. caproni.* The present study adds support to the concept that *E. caproni* and *E. paraensei* are distinct species.

A deeper understanding of how the germinal cell lineage is established and deployed in miracidia, sporocysts, and rediae is important for understanding the life cycles of digenetic trematodes as a whole. At present, there is limited and, in many cases, inconsistent information concerning germinal development. A lack of detailed description of the general nature and timing of development of germinal elements is noted in many of the classic works on digenean development. There are only a few papers specifically devoted to the examination of reproduction of sporocysts and rediae. In addition, comparative analyses of germinal development across intramolluscan stages of digenean species are rarely undertaken (Galaktionov and Dobrovolskij, 1998; Dobrovolskij et al., 2000). These studies emphasize the need for new experimental information so that a more complete understanding of germinal development can be attained.

Detailed studies of *Echinostoma caproni* intramolluscan development, in which the main focus was on sporocyst reproduction, have been undertaken (Ataev et al., 1997, 1998). It was found that germinal elements in sporocysts are represented by germinal cells (GC) and undifferentiated cells. After the transformation of miracidium to sporocyst, all GC already present, referred to as primary GC, develop into mother rediae. Some GC are subsequently derived from the proliferation of undifferentiated cells and are referred to as secondary GC (Ataev et al., 1997). It appears that only some of the secondary GC are able to complete their development. The number of embryos of mother rediae formed from secondary GC varies between sporocysts and depends on conditions of sporocyst development (Ataev et al., 1998). The pattern of germinal development in sporocysts of *E. caproni* is characterized by a high degree of stability.

The primary goal of the present study was to determine if another echinostome species, *E. paraensei,* follows a similar pattern of germinal development. Although *E. paraensei* is well-studied in some contexts, what is known relating to the germinal elements in the miracidium and early sporocyst is based only on data presented in the original description of Lie and Basch (1967). The decision to investigate the miracidium is prompted by the relative lack of variation in cellular composition throughout its free-living existence: This is a phase of relative stasis for development of germinal elements. Activation of GC is typically observed after the onset of parasitism in the snail. To facilitate comparative studies, an analysis of the number and size of germinal elements of miracidia of *E. caproni* was first made. Then a comparison was made with respect to GC in the miracidia of *E. paraensei.* An additional reason to undertake a comparative analysis of germinal material in miracidia of *E. caproni* and *E. paraensei* is because contradictory opinions exist regarding their status as distinct species (e.g., Christensen et al., 1990; Morgan and Blair, 1995, 1998; Sloss et al., 1995; Kostadinova and Gibson, 2000).

Miracidia of both *E. caproni* and *E. paraensei* were obtained from lab-reared stocks (Lie and Basch, 1967; Ataev et al., 1997). In each case, miracidia were derived from eggs that had been incubated in the dark at 26 C for 3 wk before hatching. Miracidia were fixed in Bouin's fixative. The embedding technique of Langeron (1949), appropriate for inclusion of small individuals, was used. Miracidia were first embedded in 2% gelatin previously stained with 1% neutral red. The gelatin blocks were then dehydrated and embedded in paraffin. Paraffin sections of 4–5 μm were stained with Mayer's hematoxylin or Ehrlich's hematoxylin–eosin. For ultrastructural studies, miracidia were fixed at 30 min, 1 hr, 2.5 hr, and 4 hr after hatching and were embedded in Epon 812. Sections were collected on copper grids, stained with lead citrate and uranyl acetate, and examined in a transmission electron microscope at 75 kV. Semithin sections were stained with methylene blue or toluidine blue. The Student's *t*-test was used to make statistical comparisons.

Newly hatched *E. caproni* miracidia are 120 to 128 μm long by 35 to 36 μm wide. The average number of cells per miracidium is 46.5 ± 0.6 (n = 40). Large cells are concentrated in the posterior part of the miracidium (Fig. 1). The most visible are secretory cells, whose number averaged 6.8 ± 0.2 (n = 27). They ranged in diameter from 10 to 12 μm. Unlike GC, secretory cells have large cytoplasmic processes that make determination of their cross-sectional area difficult. Therefore, their nuclear cross-sectional area was used as a characteristic feature. The cross-sectional area averaged 23.9 ± 0.6 μm^2 (n = 20). The nuclei of secretory cells contain dispersed chromatin. Large accumulations of granules of unknown nature are seen in the cytoplasm surrounding the nuclei. Areas of endoplasmic reticulum (ergastoplasm) were observed in electron microscopic preparations, indicative of the secretory function of these cells.

Germinal material is represented by GC and undifferentiated cells. GC are large cells (Fig. 1A, B) with a cross-sectional area that averaged 27.0 ± 0.8 μm^2 (n = 19). They possess a large bubble-shaped nucleus surrounded by a basophilic cytoplasm. The number of GC observed was invariably 6. Two GC located closer to the midline of the miracidium's body are larger (with a cross-sectional area of 36 μm^2) than the remaining GC. The smaller GC (with a cross-sectional area of 23 μm^2) are situated in the posterior region of the miracidium. Also, 2–3 undifferentiated cells are located in the posterior of the miracidium. These cells are relatively small in size (cross-sectional area of 17.6 ± 0.5 μm^2). Their nuclear structure is similar to nuclei of GC, but characterized by less chromatin condensation. In addition, undifferentiated cells have less distinct cytoplasmic basophilia.

Miracidia of *E. paraensei* are similar in size to those of *E. caproni* (100 to 120 μm long by 50 to 60 μm wide). Germinal material of *E. paraensei* miracidia consists of GC and undifferentiated cells (Fig. 2), and as with *E. caproni,* these cells are located in the posterior half of the miracidium. Interestingly, of the 23 *E. paraensei* miracidia examined, 3 contained an embryo, the result of GC cleavage.

The total number of *E. paraensei* miracidial cells averages 67.4 ± 1.5 (n = 20), but in specimens containing embryos, it increased to about 90. The number of secretory cells averaged 11.3 ± 0.5 (n = 22). Secretory cells are larger compared with those of *E. caproni*; their nuclear cross-sectional area is 32.3 ± 1.0 μm^2 (n = 20; $P < 0.05$). Whereas the number of GC in *E. caproni* is 6, in *E. paraensei* miracidia not containing embryos it averaged 11.6 ± 0.2 (n = 20; $P < 0.05$). The GC of *E. paraensei* miracidia are also larger than those of *E. caproni.* Their cross-sectional area averaged 36.6 ± 1.2 μm^2 (n = 28; $P < 0.05$). A predictable relation between GC size and the presence of an embryo

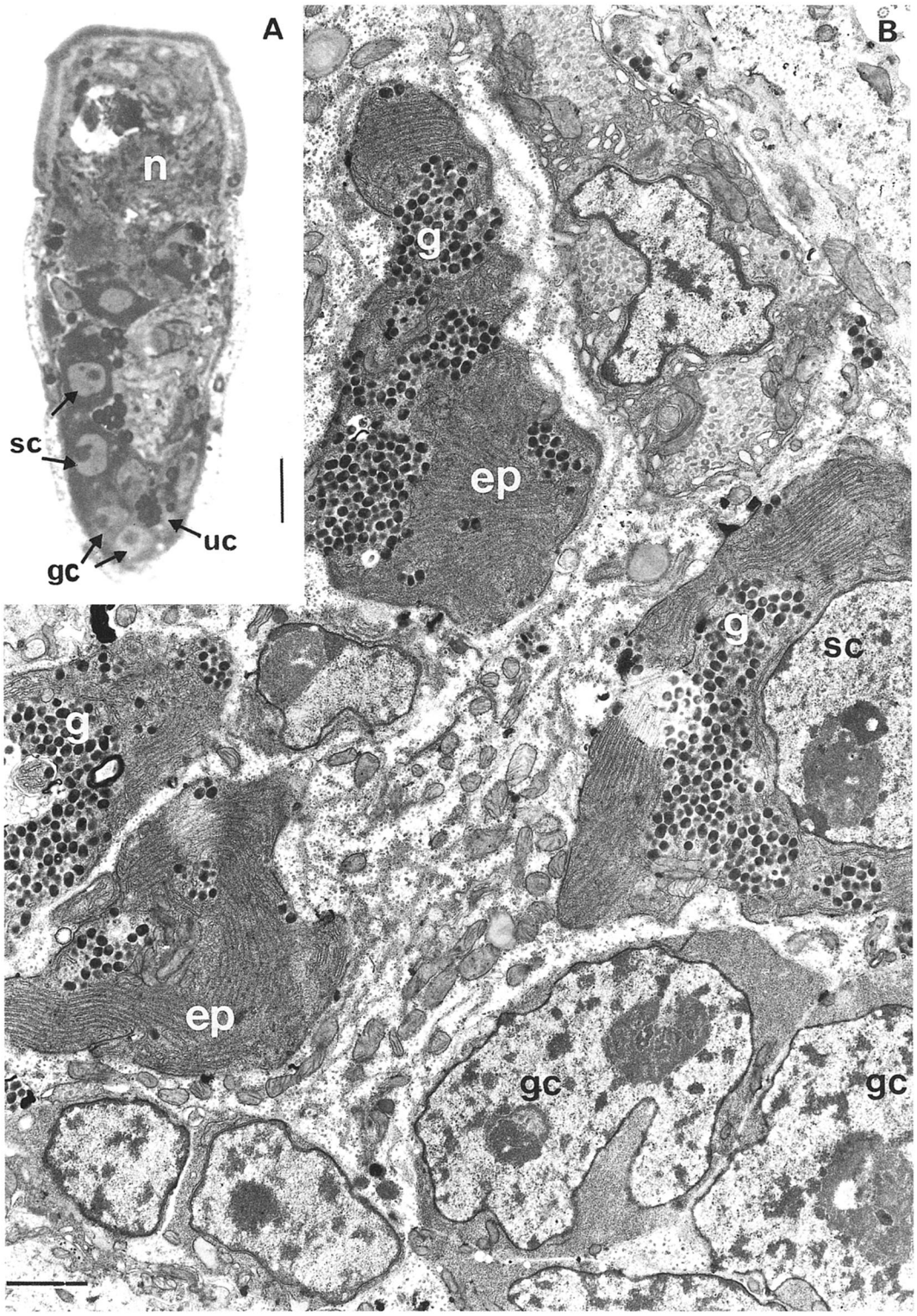

FIGURE 1. Miracidium of *Echinostoma caproni.* **A.** Longitudinal semithin section of a miracidium (gc, germinal cells; n, neural mass; sc, secretory cells; uc, undifferentiated cells). Scale bar, 10 μm. **B.** Transmission electron micrograph of a section through the posterior region of miracidium (ep, endoplastic reticulum; g, granules; gc, germinal cells; sc, secretory cells). Scale bar, 1 μm.

was not found. However, miracidia containing embryos had no more than 6–8 GC.

In the 3 miracidia observed to contain embryos, each contained only a single embryo consisting of 20–25 blastomeres (Fig. 2). Macromeres, a type of blastomere from which the embryonic tegument on the surface of the embryo is formed, were also observed. Their presence indicates that the embryo has attained the "germinal ball" stage of development.

Miracidia of *E. paraensei* contain 2–3 undifferentiated cells. They are larger than undifferentiated cells in *E. caproni.* Their cross-sectional

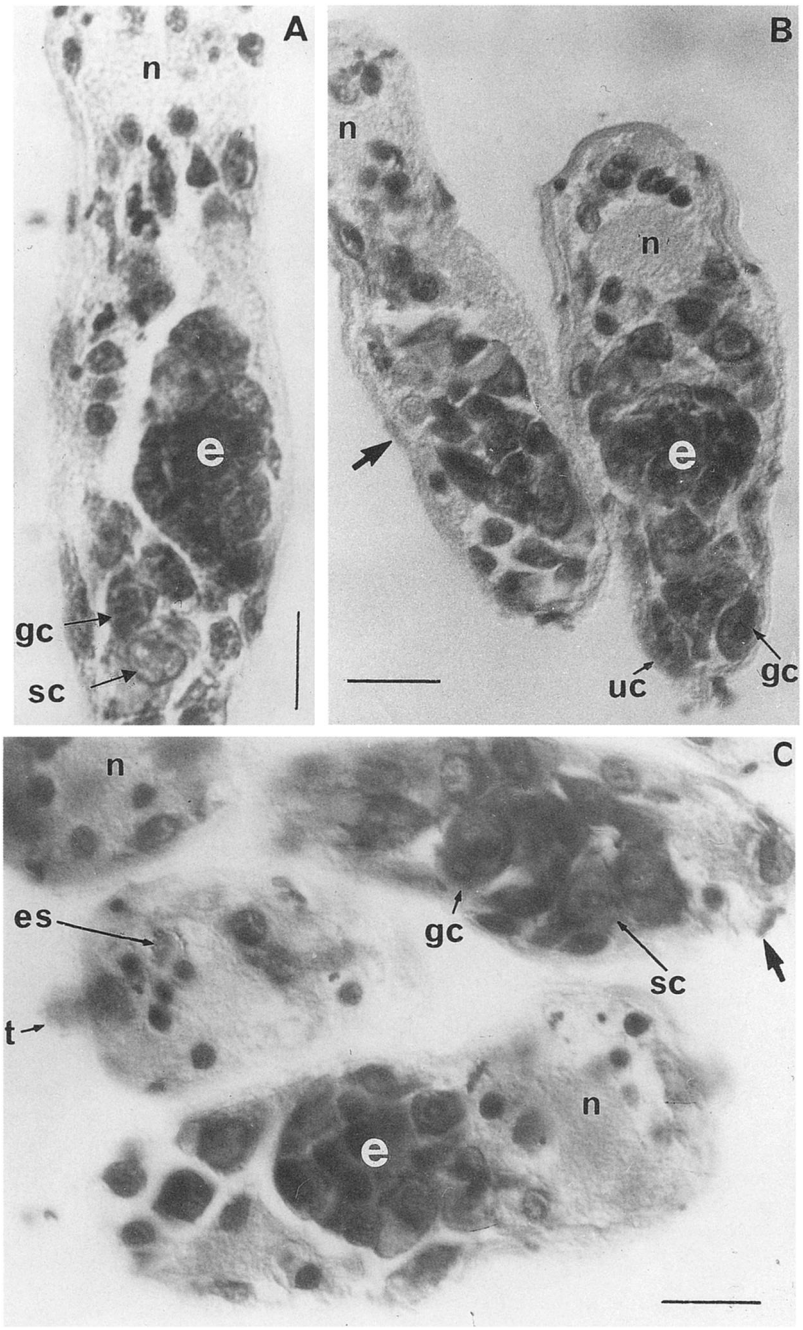

FIGURE 2. Longitudinal section of miracidia of *Echinostoma paraensei* (**A–C**) (e, embryo of mother redia; es, eye spot; gc, germinal cells; n, neural mass; sc, secretory cells; t, terebratorium; uc, undifferentiated cell). Large arrows indicate miracidia without redial embryos. Scale bars, 10 μm.

area averaged 19.6 ± 0.4 μm^2 (n = 30; $P < 0.05$). They are located in the posterior part of the miracidium (Fig. 2B).

It is important to examine carefully the composition of cells in *E. caproni* and *E. paraensei* miracidia so that germinal and somatic elements can be differentiated from one another. As a result of such scrutiny, a group of large secretory cells situated close to GC, but not destined to give rise to progeny, has been identified. The detection of these cells in the 2 echinostome species suggests that it is likely that similar cells will be found in other digenetic trematodes.

The germinal material in miracidia of both species consists of GC and undifferentiated cells. The proliferation of the latter during sporocyst development within the molluscan host gives rise to secondary GC formation. With respect to numbers of GC, *E. caproni* miracidia typically contained only 6. Miracidia of *E. paraensei* were somewhat variable in this regard, but typically contained twice as many GC as in *E. caproni.*

One or 2 GC of *E. caproni* miracidia were particularly large and began cleavage before the others, but not until the parasite had entered the snail (Ataev et al., 1997, 1998). In general, GC of miracidia of *E. paraensei* are distinctly larger than those of *E. caproni,* and are interpreted to be more fully developed. The presence of a single embryo in some *E. paraensei* miracidia is due to the tendency of 1 of the GC to

begin cleavage while the miracidium itself is still developing within the egg.

Cleavage of GC in the miracidium has also been noted in other digenean families, i.e., Transversotrematidae (Cribb, 1988), Fasciolidae (Kostova and Chipev, 1991), Allocreadidae (Peters and LaBonte, 1965; Cannon, 1971; Madhavi, 1976, 1980), Gastrothilacidae (Dinnik and Dinnik, 1960), Hemiuridae (Madhavi, 1978), and some species of Notocotylidae (Murrills et al., 1985a, 1985b). In the most extreme examples, in the so-called pedogenetic miracidia of Cyclocoelidae (Szidat, 1932) and Philophthalmidae (West, 1961; Alicata, 1962; Nollen, 1990), only 1 GC develops, into an embryo of a mother redia. In these cases, the sporocyst phase is eliminated from the life cycle and the miracidium discharges a mother redia directly in host tissues while attached to the external surface of the snail.

The tendency to form a single embryo within the miracidium of *E. paraensei* would be expected to influence the timing of reproduction after the miracidium's entry into the snail and transformation to the sporocyst stage. The relatively early release from the sporocyst of a single mother redia ahead of all others would be expected. Sapp et al. (1998) in fact noted that sporocysts of *E. paraensei* consistently release a single "precocious mother redia" at 5–6 days post infection (DPI) in snails held at 26 C. Additional mother rediae were not released until 8 DPI. The precocious mother redia is remarkable for its tendency to remain within the ventricle. With *E. caproni,* also developing in snails held at 26 C, the first embryos reach the germinal ball stage only at 3 DPI (Ataev et al., 1997), and release of the first mother rediae was observed no earlier than 8 DPI (Ataev et al., 1997, 1998). The somewhat slower development of mother rediae and lack of a precocious mother redia in *E. caproni* are consistent with the smaller GC and lack of embryos noted in the miracidia of this species.

The germinal elements of *E. paraensei* miracidia vary considerably in the timing of their development. Some primary GC are capable of initiating cleavage before completion of miracidial development. In contrast, undifferentiated cells delay their differentiation into secondary GC until after the parasite has entered the snail and has transformed into a sporocyst. For both species, the formation of GC is not confined to miracidial ontogenesis, but continues after transformation into the sporocyst stage (as undifferentiated cells divide). Consequently, the dynamics of production of mother rediae in both species is expected to be similar in that initially, embryos of rediae are formed as a result of primary GC cleavage. This is followed by the development of embryos from secondary GC. Thus, the sequence of maturation of the embryos is predetermined by the degree of maturation of the GC from which they develop. The sequential character of this reproduction may be plausibly dictated by the need to spread out progeny development so that the sporocyst does not become too large or become prematurely damaged in the process of releasing mother rediae. The issue of size of the sporocyst is important because it is typically lodged in the ventricle or aorta and can obstruct blood flow, potentially killing its host in the process. Additionally, the sporocyst lacks a birth pore (Ataev et al., 1997), so near-simultaneous release of several synchronously developing rediae could damage the sporocyst. This would potentially preclude maturation of embryos developing from secondary GC.

Differences in the number, composition, and development of germinal elements in *E caproni* and *E. paraensei* miracidia support their identity as distinct species. This view is in agreement with molecular systematics studies of Morgan and Blair (1995, 1998) and the recent review of echinostome systematics presented by Kostadinova and Gibson (2000).

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Differential Precocious Sexual Development of *Proctoeces lintoni* (Digenea: Fellodistomidae) in Three Sympatric Species of Keyhole Limpets *Fissurella* Spp. May Affect Transmission to the Final Host

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ABSTRACT: The prevalence, abundance, and developmental status of the digenetic trematode *Proctoeces lintoni* Siddiqui et Cable 1960 were compared in 3 species of keyhole limpets *Fissurella.* A total of 197 limpets was collected at Caleta Chome, south-central Chile. *Fissurella picta* and *F. costata* had the highest prevalence of infection, whereas *F. picta* showed the greatest abundance of parasites, which increased with host shell length. However, the frequency of *P. lintoni* specimens with eggs in the uterus was greatest in *F. costata.* These results suggest that an increased rate of development of a parasite in the intermediate host may shorten the residence time necessary for maturation in the final host. Thus, faster development of the parasite in *F. costata* suggests the possibility that the parasites transmitted through this host species have shorter maturation times in clingfishes than individuals transmitted via other limpet species.

The taxonomic status of species in *Proctoeces* is confusing because of the variability of characters used for discrimination (Stunkard and Uzmann, 1959; Bray and Gibson, 1980; Bray, 1983). George-Nascimento and Quiroga (1983) described *Proctoeces humboldti* (Digenea: Fellodistomidae) in limpets *Fissurella* spp. (Mollusca: Archaeogastropoda) along the coast of Chile. Later, Oliva (1984) described *P. chilensis* from the clingfish *Syciases sanguineus* (Pisces: Gobiesocidae). Subsequently, Oliva and Zegers (1988) compared *Proctoeces* specimens collected from a few limpet and clingfishes, and concluded that forms described as *P. humboldti* and *P. chilensis* are *P. lintoni* Siddiqui and Cable 1960. Since then, all records from the southeastern Pacific Ocean refer to this worm as *P. lintoni.* Further records indicate the occasional presence of this parasite in *Isacia conceptionis* and *Anisotremus scapularis* (see Oliva and Huaquín, 2000).

Ovigerous stages of *P. lintoni* are found naturally in high prevalence and abundance in the limpets and clingfishes. However, their body size, fecundity, egg size, and egg maturity are significantly larger in clingfish than in limpets. Moreover, the same pattern can be observed when they are experimentally transferred from limpets to clingfishes (George-Nascimento et al., 1998), suggesting that this clingfish species is the definitive host of *P. lintoni.*

Other studies have shown that the magnitude of infection by *P. lintoni* (cf. *Proctoeces* spp., *P. humboldti, P. chilensis*) varies among species of limpets, and between geographical locations within limpet species along the Chilean coast (Bretos and Jirón, 1980; Osorio et al., 1986; Oliva and Díaz, 1988, 1992; Oliva and Zegers, 1988; Oliva and Huaquín, 2000). No reports exist on the variation of infection between sympatric limpet species. Such variations may be affecting the dynamics of transmission from each limpet species to the clingfishes.

Proctoeces lintoni shows a high degree of reproductive development in *Fissurella* spp. (George-Nascimento and Quiroga, 1983; Oliva and Zegers, 1988; George-Nascimento et al., 1998). *Proctoeces* species have been shown to become ovigerous in invertebrate hosts (Wardle, 1980;

TABLE I. Prevalence, abundance, body length, presence of eggs in *Proctoeces lintoni* of 3 species of *Fissurella* limpets, and correlations between some variables.

	F. picta	*F. costata*	*F. maxima*
Prevalence	96 (A)*	96 (A)	58 (B)
Abundance (mean ± SD)	11.2 ± 13.3 (A)	4.3 ± 2.8 (B)	6.6 ± 12.3 (B)
Host shell length (cm) (mean ± SD)	5.6 ± 0.9 (B)	5.5 ± 1.0 (B)	6.5 ± 1.3 (A)
Parasite body length (mm) (mean [range])	3.7 (1.5–4.6) (A)	3.9 (2.1–5.5) (A)	3.5 (2.6–4.2) (A)
% Parasites with eggs	49 (B)	83 (A)	42 (B)
Spearman's correlation Abundance vs. shell length	0.59†	0.07	0.11
Spearman's correlation Parasite length vs. shell length	0.65 (A)†	0.75 (A)†	0.86 (A)†
Number of parasites	83	24	33
Number of hosts	119	25	53

* Values with the same letter in a row are not significantly different ($P > 0.05$).
† Spearman's correlation significant.