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# EMBRYONIC DEVELOPMENT, FEEDING AND INTRACAPSULAR OXYGEN AVAILABILITY IN *TROPHON GEVERSIANUS* (GASTROPODA: MURICIDAE)

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## ABSTRACT

Large-scale patterns of encapsulated embryo development and causal factors determining developmental success in the marine environment have been relevant issues of research for decades. We studied the embryonic development and intracapsular oxygen availability of *Trophon geversianus* in egg capsules from northern Patagonia (Golfo Nuevo, Argentina). The intracapsular embryonic development had a mean duration of 112 days (at 12–14°C). The initial number of eggs per capsule was 197 (mean diameter 270 μm), but on average only four embryos per capsule reached the juvenile stage. Embryos fed on nurse eggs (which disintegrated spontaneously 48 h after oviposition). The sequence of intracapsular developmental stages was recorded from egg to hatching occurred at the crawling stage (mean shell length 2.8 mm). Maximum growth rate was observed at the 'veliger' stage, while nurse eggs were consumed. A decrease in embryo number was observed between prehatching and hatching. Cannibalism was recorded in an egg capsule containing a prehatching embryo, which showed signs of shell drilling by the accessory boring organ. Intracapsular oxygen availability decreased from 90% during the cleavage stage to 45% of air saturation in the final stages of development (at 13°C). Abnormal embryos were recognized in egg capsules, probably associated with food competition or oxygen limitation.

## INTRODUCTION

The Muricidae are an important family of marine gastropods from both ecological and economic perspectives (Benkendorff, Westley & Gallardo, 2004; Vasconcelos *et al.*, 2008). This family includes many predators that play key roles determining community structure (Durán & Castilla, 1989; Fairweather & Underwood, 1991; Morton, 1999). Many relatively large species are harvested commercially, supporting important coastal fisheries (Castilla & Defeo, 2001; Michel-Morfin, Chávez & González, 2002; Vasconcelos *et al.*, 2008). In the southwestern Atlantic Ocean, Muricidae are important components of neogastropod diversity, in particular the genus *Trophon* Monfort, 1810, which includes at least 15 species (Pastorino, 2005). In spite of the importance of this genus, biological knowledge of many species is extremely poor (Pastorino, 2005). *Trophon geversianus* (Pallas, 1774) shows the widest geographic distributional range and inhabits both rocky intertidal and shallow subtidal zones. In the southwestern Atlantic this species is reported from 35°S to 56°S latitude, including the Malvinas (Falkland) Islands, while on the southeastern Pacific coast it ranges from 42°S to 56°S (de Castellanos & Landoni, 1993; Griffin & Pastorino, 2005; Pastorino, 2005). Adults can reach up to 100 mm in shell length (Harasewych, 1984; de Castellanos & Landoni, 1993; Pastorino, 2005).

Previous studies on *T. geversianus* in the southwestern Atlantic have focused on its taxonomy and anatomy (Pastorino, 2005), and some reproductive aspects including spawn characteristics, hatching modality and reproductive seasonality (Zaixso, 1973; Penchaszadeh, 1976; Cumplido, Averbuj & Bigatti, 2010). On the southeast Pacific coast, previous research has contributed substantial knowledge concerning reproductive biology, ecology and culture (Guzmán *et al.*, 1997; Chávez & Medina, 1998; Santana, 1998; Santana & Cañete, 2001; Gallardo, Manque & Filún, 2004; Andrade, 2006; González *et al.*, 2007).

*Trophon geversianus* is a gonochoristic species with internal fertilization. Females deposit a variable number of erect, discoidal egg capsules on hard substrates (Pastorino, Penchaszadeh & Scarabino, 2007; Cumplido *et al.*, 2010). Embryos are surrounded by numerous nurse eggs used as nutritional resources during intracapsular development and eventually hatch as crawling juveniles (Zaixso, 1973; Penchaszadeh, 1976). In the intertidal zone the egg capsules are exposed to temperature variations in water and air during semidiurnal tides (Cumplido *et al*., 2010), which may impose some limitations on embryo development (Strathmann & Strathmann, 1995). Limitations on oxygen diffusion constrain the size and shape of aggregations of embryos in egg masses or egg capsules, affecting the number of eggs that females assign to each capsule as well as the costs of parental care (Perron & Corpuz, 1982; Strathmann & Strathmann, 1995; Fernández *et al*., 2007; Moran & Woods, 2007). In addition, low oxygen availability affects the development of embryos, size at hatching and calcification (Booth, 1995; Strathmann & Strathmann, 1995; Cancino, Gallardo & Torres, 2003; Fernández, Pappalardo & Jeno, 2006). Furthermore, it has been hypothesized that oxygen limitation determines the number of developing embryos during gastropod early development (Lardies & Fernández, 2002).

In this paper we describe, under laboratory conditions, the embryonic development, embryonic feeding and intracapsular oxygen availability of *T. geversianus* from Golfo Nuevo, Argentina.

## MATERIAL AND METHODS

Thirty individuals of *Trophon geversianus* (19 females and 11 males) were collected from the rocky intertidal of Punta Cuevas, Golfo Nuevo (Puerto Madryn, Chubut, Argentina) (42°46′37″S, 64°59′51″W) in December 2007 and placed in aquaria with aerated seawater at controlled temperature (12–14°C). Initially, the snails were acclimatized for 2 weeks using a photoperiod of 15:9 h light:dark, corresponding to summer conditions in the southern hemisphere. Later, this photoperiod was gradually decreased to winter conditions (9:15 h light:dark), reducing the light interval by increments of 30 min each week. In the aquaria the snails were fed *ad libitum* with mussels collected from the intertidal. Oviposition events were recorded every day. Only recently laid egg capsules were used to study embryo development.

### Development

From a total of 98 egg capsules that were laid on the glass walls of the aquaria, 68 egg capsules were randomly selected to analyse developmental stages over time. Embryos within the egg capsules were classified into eight developmental stages, from egg to miniature adult (Table 1). Thirty egg capsules were randomly selected and maintained in aquaria until hatching in order to study the total developmental time. Intracapsular egg diameter was measured before cellular cleavage and mean size of 50 eggs per capsule was calculated. Although the cell cleavage stage was difficult to observe, all cleaved eggs were measured within the egg capsules. From early ‘veliger’ to the hatching stages, all embryos were measured and the total number of embryos in each capsule counted. All measurements were conducted using a stereomicroscope with a 0.1 mm ocular micrometer. The number of embryos per egg capsule was compared among different developmental stages (from ‘veliger’ stage) using a one-way ANOVA. We excluded the early ‘veliger’ stage from the statistical analysis because of its low replication ($n = 2$). Sizes of eggs and embryos were compared between developmental stages using a one-way ANOVA. *A posteriori* Tukey tests were used to determine significant differences between treatments.

### Intracapsular oxygen availability

In order to assess intracapsular oxygen availability during development, we monitored air saturation inside the capsules using a microsensor. Egg capsules of *T. geversianus* were collected in the intertidal zone of Punta Cuevas (Golfo Nuevo) and maintained in the laboratory under the same conditions as above (12–14°C, in aerated seawater) for a minimum of 24 h before measurements. Five embryo stages were used (egg, ‘veliger’, late ‘veliger’, pediveliger and prehatching). Intracapsular oxygen availability (% air saturation) was measured in the laboratory using a needle-type fibre-optic microsensor connected to a Microx TX3 oxygen meter (PRESENS). Calibration to 0 and 100% air saturation was done at 13°C using $Na_2SO_3$-saturated sea water and aerated sea water, respectively. The microsensor was inserted through a small hole punctured with a syringe in the exit plug area. The tip of the fibre-optic was placed in the centre of the capsule. Measurements were done while the capsules were submerged in air-saturated seawater at constant temperature 13°C. Measurements were continued for 30 min, to avoid possible measurement errors due to drift or to oxygen exchange through the measurement hole. However, only the recordings during the 10 central minutes were used for statistical tests, to avoid possible errors related to manipulation of the egg capsule at the beginning and end of the recording time. The average oxygen availability (estimated as % air saturation) per capsule was calculated. At least four replicates per developmental stage were carried out. Mean oxygen availability was compared between developmental stages using a one-way ANOVA and *a posteriori* Tukey tests were used to determine significant differences between treatments.

**Table 1.** Embryonic intracapsular stages of *Trophon geversianus* at Punta Cuevas, Golfo Nuevo.

| Stage | Description | Time* (days) | Mean embryos size (mm) | Mean embryos per capsule | Number of egg capsule (*n*) |
|---|---|---|---|---|---|
| 0 (egg) | Round and whitish, before cellular cleavage | 0–1 | 0.27 ± 0.01 (0.24–0.32) | 197.60 ± 14.88$^{\dagger}$ | 5 |
| 1 (cell cleavages) | Embryo with 2–8 macromeres and micromeres | 1–12 | 0.28 ± 0.01 (0.25–0.37) | 11.83 ± 0.01$^{\dagger}$ | 6 |
| 2 (early ‘veliger’) | Embryo with incipient velum, mouth and empty stomach | 12–20 | 0.42 ± 0.03 (0.40–0.46) | 9.00 ± 2.00 | 2 |
| 3 (‘veliger’) | Embryo with ciliated velum and conspicuous stomach full of eggs | 20–30 | 1.16 ± 0.08 (0.93–1.42) | 8.17 ± 1.49 | 6 |
| 4 (late ‘veliger’) | Large embryo with developed velum and large ‘prismatic’ cells | 30–50 | 1.64 ± 0.04 (1.39–1.99) | 6.14 ± 1.19 | 14 |
| 5 (pediveliger) | Embryo with developing foot; velum reduced | 50–70 | 1.92 ± 0.08 (1.52–2.43) | 5.23 ± 0.79 | 13 |
| 6 (prehatching) | Juvenile with calcified shell and developed foot; velum resorbed | 70–100 | 2.23 ± 0.08 (1.90–2.39) | 4.30 ± 0.30 | 10 |
| 7 (hatching) | Crawl-away juveniles | 100–120 | 2.79 ± 0.04 (2.61–3.05) | 4.17 ± 1.33 | 12 |

Values represent mean size ± SE and range in parentheses. *Developmental time recorded in aquarium. $^{\dagger}$Embryos were unable to be recognized before cellular cleavage, therefore we count and measured eggs (in general) for the first two developmental stages.

## RESULTS

### *Development*

The egg capsules of *Trophon geversianus* had on average 197.6 ± 14.88 (mean ± SE) uncleaved eggs (Table 1; Fig. 1A), with a range of 152–240 eggs, embedded in a transparent gelatinous matrix. The first cellular cleavages occurred within the first 24 h after egg laying. However, this process was observed in few eggs within each egg capsule. These eggs followed a typical holoblastic spiralian division; stages from morula to 'trochophore' were not observed. After 48 h the nurse eggs began to disintegrate within the egg capsule (Fig. 1B). Sizes of eggs and

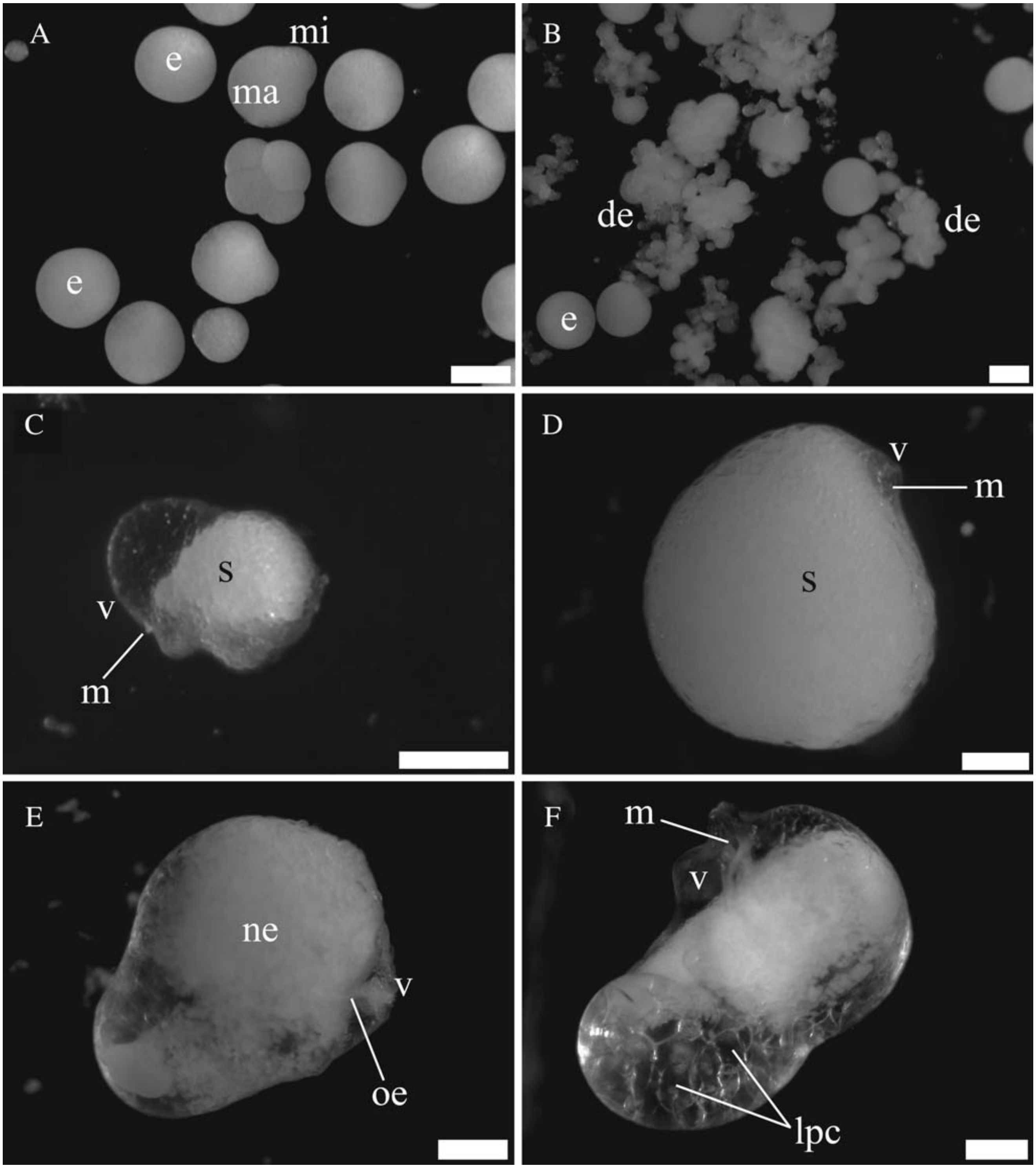

**Figure 1.** Embryological development of *Trophon geversianus*. **A.** Eggs before first cleavage and cellular cleavage stage. **B.** Eggs and disaggregated nurse eggs. **C.** Early 'veliger' stage with mouth, incipient velum and very few nurse eggs inside stomach. **D, E.** 'Veliger' stage embryo full of eggs. **F.** Late 'veliger' stage embryo with developing velum, large 'prismatic' cells and digested nurse eggs. Abbreviations: e, eggs; de, disaggregated eggs; lpc, large 'prismatic' cells; ma, macromere; mi, micromere; m, mouth; ne, nurse eggs; oe, oesophagus; s, stomach; v, velum. Scale bars = 200 μm.

embryos were significantly different between developmental stages, steadily and significantly increasing from early 'veliger' to hatching stage ($F_{7,60}= 197.01$; $P < 0.001$; Fig. 5A). The early 'veliger' stage was characterized by the presence of a mouth and the development of the velum, with very few nurse eggs inside the stomach (Fig. 1C). A two-fold increase in mean size was observed at the 'veliger' stage (Table 1; Figs 1D, E, 5A) when intracapsular embryos began to ingest the disintegrated nurse-egg particles, manipulating them with the ciliated velar lobes. The embryos are transparent, which allowed observation of the ingestion of a large amount of disintegrated nurse eggs. The transition from the 'veliger' to the late 'veliger' stage

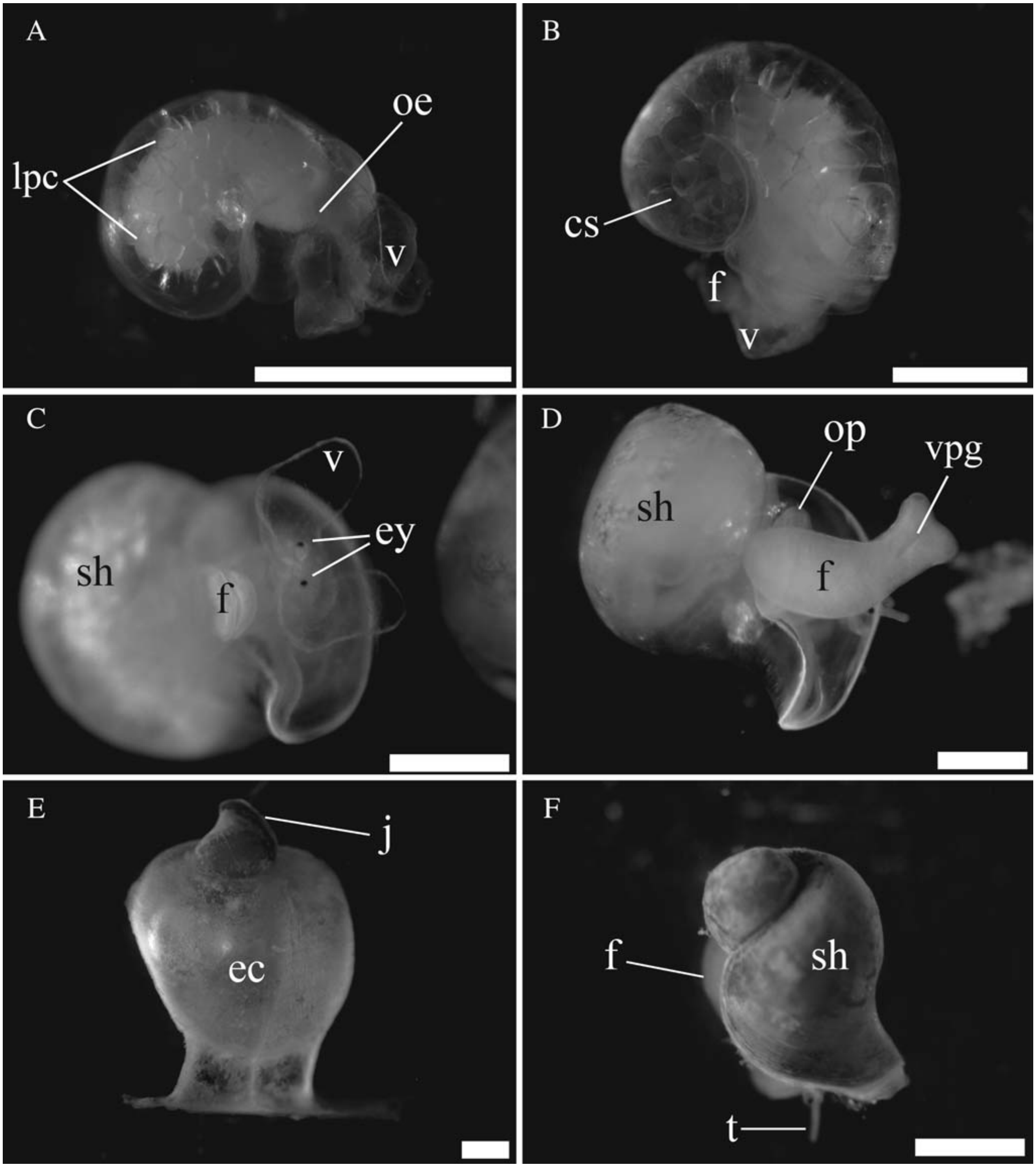

**Figure 2.** Embryological development of *Trophon geversianus* (continued). **A.** Late 'veliger' stage with developed velum and large 'prismatic' cells. **B, C.** Pediveliger stage embryo with coiled-shell, eyes and developing foot. **D.** Prehatching stage embryo with developed foot and ventral pedal glands, probably containing the ABO. **E.** Juvenile hatching from capsule plug. **F.** Hatchling. Abbreviations: cs, coiling shell; ec, egg capsule; ey, eyes; f, foot; lpc, large 'prismatic' cells; j, juvenile; oe, oesophagus; op, operculum; sh, shell; t, tentacle; v, velum; vpg, ventral pedal glands. Scale bars = 1 mm.

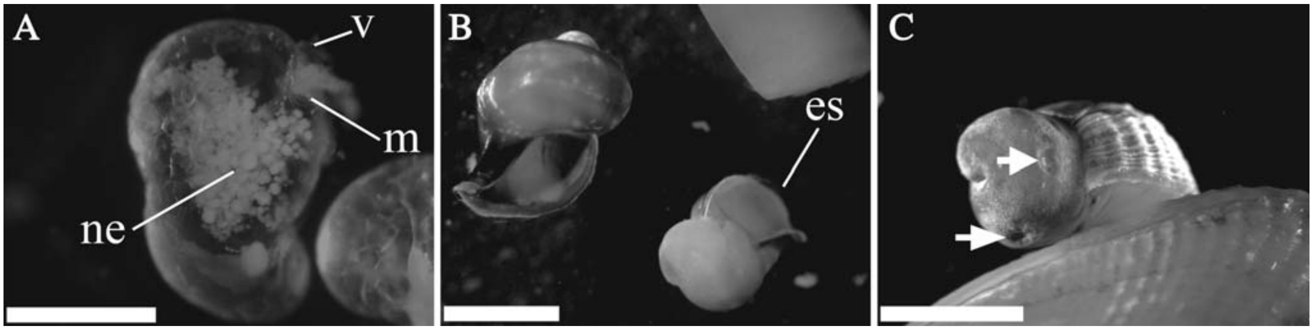

**Figure 3.** Intracapsular nutrition of *Trophon geversianus*. **A.** Adelphophagy: late 'veliger' embryo feeding on nurse eggs. **B.** Cannibalism: empty embryonic shell (of smaller size) probably ingested by a prehatching embryo. **C.** Cannibalism evidence: this hatchling survived drilling attempts (arrows) by other embryos during intracapsular development (note that snail is probably drilling the mussel shell on which it sits). Abbreviations: es, empty shell, ne, nurse eggs; m, mouth; v, velum. Scale bars = 1 mm.

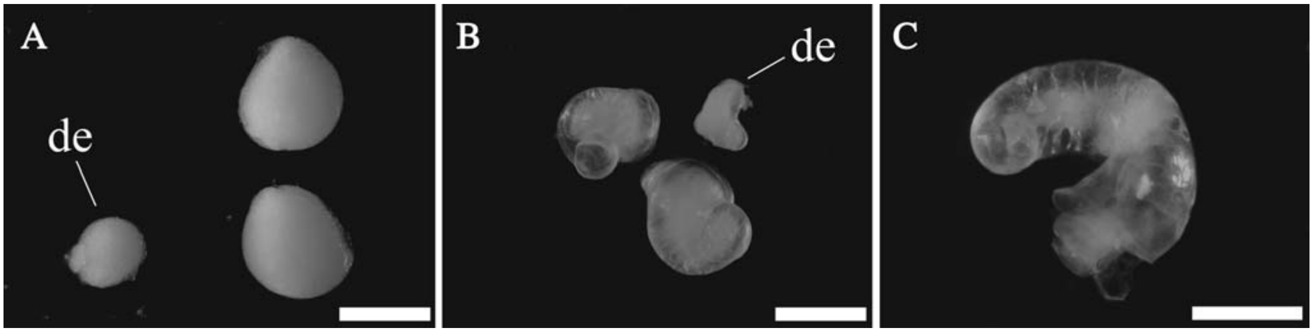

**Figure 4.** Intracapsular competition and malformed embryos of *Trophon geversianus*. The difference in size at the same stage is clearly shown here: **A.** 'Veliger' stage. **B.** Pediveliger stage. **C.** Abnormal pediveliger embryo without shell. Abbreviation: de, delayed embryo. Scale bars = 1 mm.

was characterized by the elongation of the embryo and subsequent coiling, and by further increase in size, with large 'prismatic' cells covering the stomach and the posterior end of the embryo (Table 1; Figs 1F, 2A, 5A). The digestion of the nurse-egg particles occurred at the same time that a translucent shell developed. The food particles were trapped in mucus and carried to the mouth (Fig. 3A). Embryo size also significantly increased from late 'veliger' to pediveliger stage (Table 1; Fig. 5A); the latter was characterized by reduction of the velum, enlargement of the cephalic tentacles with basal eyes and formation of the foot. The embryonic shell grew as the foot developed and the velum disappeared (Table 1; Fig. 2B, C). The prehatching embryo was characterized by the absence of the velum, a completely formed foot with an operculum, differentiation of the pedal sole tissue in the form of a split that could correspond to the accessory boring organ (ABO), and coloured embryonic shell with yolk reserves in the apical section (Fig. 2D).

The mean number of embryos per egg capsule showed a more than two-fold decrease from early stages (early 'veliger') to embryos in later stages of development (Table 1; Fig. 5B), although the statistical differences showed only a marginal *P*-value ($F_{4,50} = 2.33$; $P = 0.069$). Nevertheless, intracapsular cannibalism was supported by direct evidence in the prehatching stage. In two egg capsules (3%) we found empty embryonic shells together with larger prehatching live embryos (Fig. 3B), and also observed embryonic shells with signs of drilling, with a typical hole formed by the ABO (Fig. 3C).

After 100–120 days of intracapsular development, the crawling juveniles emerged through a plug situated at the top of the egg capsule (Fig. 2E, F). The hatching juveniles exhibited a purple, calcified shell. On average only 2.1% of the initial number of eggs developed successfully. Between two and six embryos hatched per egg capsule, reaching a mean shell length of 2.79 ± 0.03 mm, which represents a 10-fold increase in size from the egg stage (Table 1). Size differences among the embryos within each egg capsule were observed (Fig. 4A, B); malformed embryos were recorded in only three capsules (<5%) (Fig. 4C).

### *Intracapsular oxygen conditions*

Mean intracapsular oxygen availability (% air saturation) showed significant changes during development ($F_{15,4} = 8.20$; $P < 0.001$; Fig. 5C). Intracapsular air saturation ranged from 90% during the cleavage and 'veliger' stages to 45% at the prehatching stage, while 100% air saturation was recorded in the water of the experimental aquaria in all cases (extracapsular environment). Intracapsular air saturation remained high and relatively constant between egg and late 'veliger' stages, decreasing significantly at prehatching stage, with intermediate values in the pediveliger stage (Fig. 5C).

## DISCUSSION

This is the first detailed study of intracapsular development, nutrition and oxygen availability of *Trophon geversianus* from Atlantic waters under laboratory conditions. To our knowledge, this is also the first report of intracapsular cannibalism by drilling, with probable use of the ABO.

Species that complete their development in protective structures are provided with food in different ways: intracellular yolk, nurse eggs, intracapsular fluids or protein from the capsule walls (Rivest, 1983; Miloslavich, 1999; Ojeda & Chaparro, 2004). Several possible modes of nurse-egg ingestion for neogastropods were suggested by Fioroni (1967), including

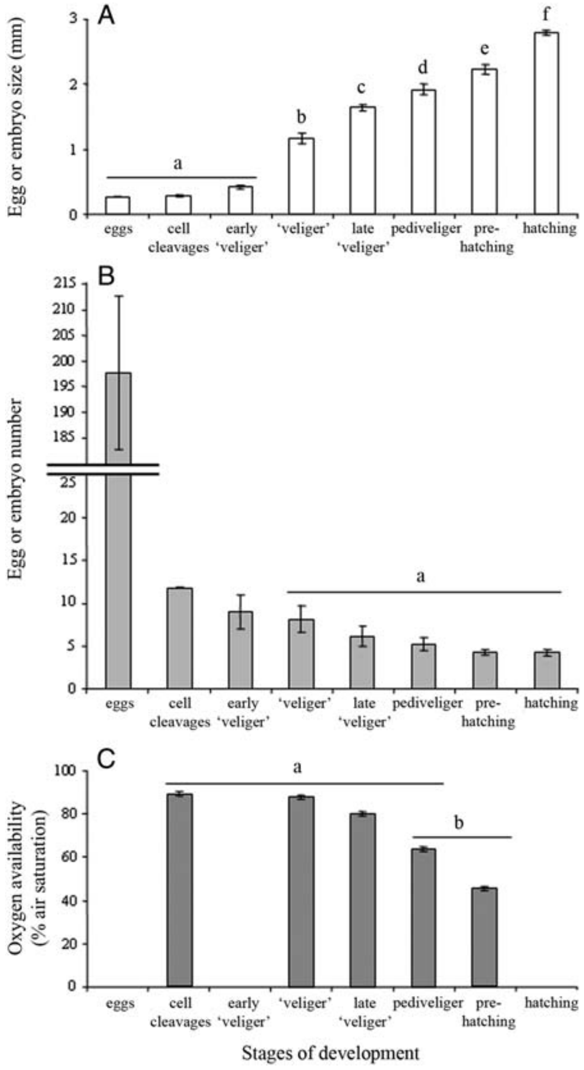

**Figure 5.** Statistical analyses among developmental stages of *Trophon geversianus*. **A.** Mean egg or embryo size. **B.** Mean egg or embryo number. **C.** Mean intracapsular oxygen availability (% of air saturation). Bars show mean ±SE. Bars not connected by the same letter are significantly different.

degradation by rotation of the nurse eggs, embryonic mechanical destruction of the eggs and ingestion of the particles, and ingestion of entire nurse eggs. In the case of *T. geversianus* intracapsular development seems to be achieved by the ingestion of disintegrated nurse eggs (Penchaszadeh, 1976). In this work we observed the ingestion of a large amount of nurse eggs after their spontaneous disintegration within the egg capsules, 48 h after spawning.

The number of eggs per capsule (mean 197) was lower and the intracapsular developmental time shorter (100–120 days) than was observed for another intertidal population of *T. geversianus* studied in the Magellan Strait (mean 1450 eggs; development time 120–150 days) on the southern Pacific coast (51°30′S, 70°51′W) by Santana (1998). The lower number of eggs may be associated with the smaller female sizes and smaller egg capsules in the Punta Cuevas population (Cumplido *et al*., 2010). However, the proportion of embryos that develop (estimated as the ratio between number of hatchlings and number of initial eggs) is similar between both populations: 4/197 = 0.020 in Punta Cuevas (Table 1) *vs* 19/1,450 = 0.013 in the Magellan Strait (P.P., unpubl.). The differences in number of eggs and developmental time may be associated with differences in climatic parameters related to latitudinal gradients, which could induce different metabolic responses.

The mean number of embryos per capsule decreased more than two-fold from the 'veliger' to the prehatching stage (Fig. 5B), coinciding with a 10-fold increase in embryonic size (Fig. 5A). The main increase in size occurred between early 'veliger' and 'veliger' stage, which can be explained by the development of a mouth and the ingestion of a large amount of nurse-egg particles facilitated by the ciliary currents of the velar lobes. The decreasing number of embryos throughout development could be a result of intracapsular cannibalism triggered by competition for limiting resources, such as food. Intracapsular cannibalism represents an additional nutritional resource inside the egg capsule, avoiding the disadvantages of sharing/competing for the supply of nurse eggs (Averbuj & Penchaszadeh, 2010). Cannibalism is a significant process in *T. geversianus*, supported by our observations of prehatching individuals with signs of drilling on their shells, a clear proof of cannibalistic behaviour. Located on the foot sole of *T. geversianus* (Harasewych, 1984), the ABO is common to all Muricidae (Gruber & Carriker, 1990; Carriker & Gruber, 1999). The ABO is a compact structure, a histologically, physiologically and biochemically complex gland by which boring predatory gastropods penetrate the calcareous shells of live molluscan prey (Carriker & Gruber, 1999). A different tissue was observed at the midline of the foot sole of *T. geversianus* at the prehatching stage and is identifiable as the ventral pedal glands containing the ABO. Hatchlings of *T. geversianus* are more resistant to starvation than juveniles of other muricid species. This is possibly due to prolonged ingestion of yolk in the intracapsular fluid throughout the long developmental period (Gallardo *et al*., 2004). Here we show that reserves can also be obtained from sibling cannibalism in the later stages of development. Development of large individuals supported by feeding on nondeveloping nurse eggs (adelphophagy) or on siblings (cannibalism) has been reported in some vermetids (Miloslavich & Penchaszadeh, 1992; Calvo & Templado, 2004; Strathmann & Strathmann, 2006), calyptraeids (Miloslavich & Penchaszadeh, 2001) and nassariids (Averbuj & Penchaszadeh, 2010).

Another limiting resource during intracapsular development is oxygen (Strathmann & Strathmann, 1995; Cancino *et al*., 2000; Lardies & Fernández, 2002; Fernández *et al*., 2006). Previous studies have suggested that limiting oxygen conditions could affect developmental rate and survival, determining the ratio between initial number of eggs and final developed embryos (Lardies & Fernández, 2002). Egg capsules of *Acanthina monodon* incubated under hyperoxia showed an increase in the number of final developed embryos, suggesting that oxygen limitation could be determining developmental success (Lardies & Fernández, 2002). Intracapsular oxygen conditions in *T. geversianus* in prehatching stages were similar to those of *A. monodon* (at a similar temperature range) and also to those of *Crepidula coquimbensis* (Fernández *et al*., 2006; Brante, Fernández & Viard, 2008). Low oxygen levels were observed during late embryo development of *T. geversianus*. Thus, intracapsular oxygen limitation may not only affect the final number of embryos that develop successfully, but could also be relevant for the proper development of the embryos. In fact, slightly higher intracapsular oxygen conditions in *Chorus giganteus* (55% oxygen saturation) produced low calcification and failures in hatching (Cancino *et al*., 2000). The presence of malformed embryos in *T. geversianus* egg capsules could be a response to oxygen limitations or competition between developing embryos. It is yet unclear if slower-developing embryos or malformed embryos, as a by product of poor intracapsular oxygen conditions, could be preyed upon more easily, increasing cannibalism among siblings.

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