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Biosynthesis in the Albumen Gland-Capsule Gland Complex Limits Reproductive Effort in the Invasive Apple Snail *Pomacea canaliculata*

M. P. CADIERNO[1,*], L. SAVEANU[2,*], M. S. DREON[1,3], P. R. MARTÍN[2], AND H. HERAS[1,4,†]

[1]*Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)–Universidad Nacional de La Plata (UNLP), 60 y 120, (1900) La Plata, Argentina;* [2]*Laboratorio de Ecología, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur (UNS), San Juan 670, (8000) Bahía Blanca, INBIOSUR (UNS-CONICET), Argentina;* [3]*Cátedra de Bioquímica y Biología Molecular, Facultad de Cs. Médicas, UNLP, 60 y 120, (1900) La Plata, Argentina; and* [4]*Cátedra de Química Biológica, Facultad Cs. Naturales y Museo, UNLP, 60 y 121, (1900) La Plata, Argentina*

Abstract. High fecundity often contributes to successful invasives. In molluscs, this may be facilitated by the albumen gland-capsule gland complex, which in gastropods secretes the egg perivitelline fluid that nourishes and protects embryos. The biochemistry of the albumen gland-capsule gland complex and its relationship with fecundity remain largely unknown. We addressed these issues in *Pomacea canaliculata* (Lamarck, 1822), a highly invasive gastropod whose fecundity and reproductive effort exceed those of ecologically similar gastropods. We evaluated the dynamics of its major secretion compounds (calcium, polysaccharides, and total proteins) as well as the gene expression and stored levels of perivitellins during key moments of the reproductive cycle, that is, before and after first copulation and at low, medium, and high reproductive output. Copulation and first oviposition do not trigger the onset of albumen gland-capsule gland complex biosynthesis. On the contrary, soon after an intermediate reproductive effort, genes encoding perivitellins overexpressed. A high reproductive effort caused a decrease in all albumen gland-capsule gland complex secretion components. Right after a high reproductive output, the albumen gland-capsule gland complex restored the main secretion components, and calcium recovered baseline reserves; but proteins and polysaccharides did not. These metabolic changes in the albumen gland-capsule gland complex after multiple ovipositions were reflected in a reduction in egg mass but did not compromise egg quality. At the end of the cycle, egg dry weight almost doubled the initial albumen gland-capsule gland complex weight. Results indicate that albumen gland-capsule gland complex biosynthesis limits a constantly high reproductive output. Therefore, lowering fecundity by targeting biosynthesis could effectively reduce the rate of this species' spread.

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* These authors contributed equally.

† To whom correspondence should be addressed. E-mail: h-heras@med.unlp.edu.ar.

Abbreviations: AC, after copulation; *ACTB*, β-actin; AFO, after first ovipositon; AFP, after first pause in oviposition; AG, albumen gland; AG-CG, albumen gland-capsule gland complex; ARO, after repeated ovipositions; BC, before copulation; BM, before maturity; CG, capsule gland; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; NRT, no-retrotranscription control; NTC, no-template control; PVF, perivitelline fluid.

Introduction

Biological invasions pose a serious threat to ecosystems worldwide, being effectively irreversible. Some species introductions degrade human health, while others cause economic losses or drive losses in the biological diversity of native species (Vitousek *et al.*, 1997; Sala *et al.*, 2000). The freshwater gastropod *Pomacea canaliculata* (Lamarck, 1822) has all the above-mentioned characteristics. This South American apple snail (family Ampullariidae) has been introduced to Southeast Asia, North America, Spain, and some Pacific islands (Cowie, 2002; Rawlings *et al.*, 2007; EFSA, 2014). It is currently listed as one of the 100 worst invasive species in the world (Lowe *et al.*, 2000), being responsible both for ecolog-

ical impacts to wetlands and agricultural damage to aquatic crops, such as rice and taro (Horgan *et al.*, 2014), and for significant economic losses (Nghiem *et al.*, 2013). In some of the introduced areas, it was additionally associated with eosinophilic meningoencephalitis outbreaks, because it is an intermediate host of the nematode *Angiostrongylus cantonensis*, the parasite that causes this potentially fatal human disease (Lv *et al.*, 2009).

The trophic flexibility of *P. canaliculata* (Saveanu *et al.*, 2016) and the plasticity of some life-history traits (Estebenet and Martín, 2002; Tamburi and Martín, 2009b, 2011) are related to the remarkable ability of this species to adapt to new environments in invaded areas. Fecundity is also a good predictor of the negative impacts of freshwater molluscs on human activities and on ecosystem functions (Keller *et al.*, 2007). In this regard, females of *P. canaliculata* exhibit great fecundity in being able to lay from 1300 to 11,000 eggs during their lifespan under laboratory conditions (4500, on average; Martín and Estebenet, 2002). During the reproductive season, which lasts from November to March in southern South America (Fig. 1A; Martín *et al.*, 2001), females deposit 2–3 egg masses per week above the water surface, consisting of 100–300 eggs with a calcareous eggshell (Winik and Castro-Vazquez, 2015). After a single copulation, females can lay thousands of eggs during several months (Albrecht *et al.*, 1996; Burela and Martín, 2011). However, both males and females are highly promiscuous, copulating repeatedly with the same partner or different partners (Albrecht *et al.*, 1996; Burela and Martín, 2011).

Many aspects of the life cycle in *P. canaliculata* show great inter- and intrapopulation variation. For instance, age at maturity under controlled conditions varies within the same population (Martín and Estebenet, 2002), mainly depending on food availability and genetic variation, because variability in age at maturity mostly disappears when full siblings are reared under a strictly controlled diet (Tamburi and Martín, 2009a). The number of eggs per egg mass is another highly variable trait, even for the same female (Estebenet and Cazzaniga, 1992; Albrecht *et al.*, 1996). The frequency of oviposition is also variable in *P. canaliculata*: several egg masses can be laid in consecutive days or with a pause of a few days in between, with or without new copulations (Fig. 1A); in some instances two small egg masses are laid in the same night.

The reproductive effort of *P. canaliculata* is higher than that of other gastropods with similar life cycles (Estebenet and Cazzaniga, 1993; Estoy *et al.*, 2002b). For instance, a single egg mass represents 20% of a female's volume (Fig. 1B), but the estimated reproductive output along one reproductive season is twice a female's volume (Estebenet and Cazzaniga, 1993). Irrespective of food availability, about 90% of the female somatic production on a weight basis is allocated to eggs (Estoy *et al.*, 2002a). Many of these characteristics of *P. canaliculata* reproduction can be understood by looking at the anatomical and molecular levels of egg production.

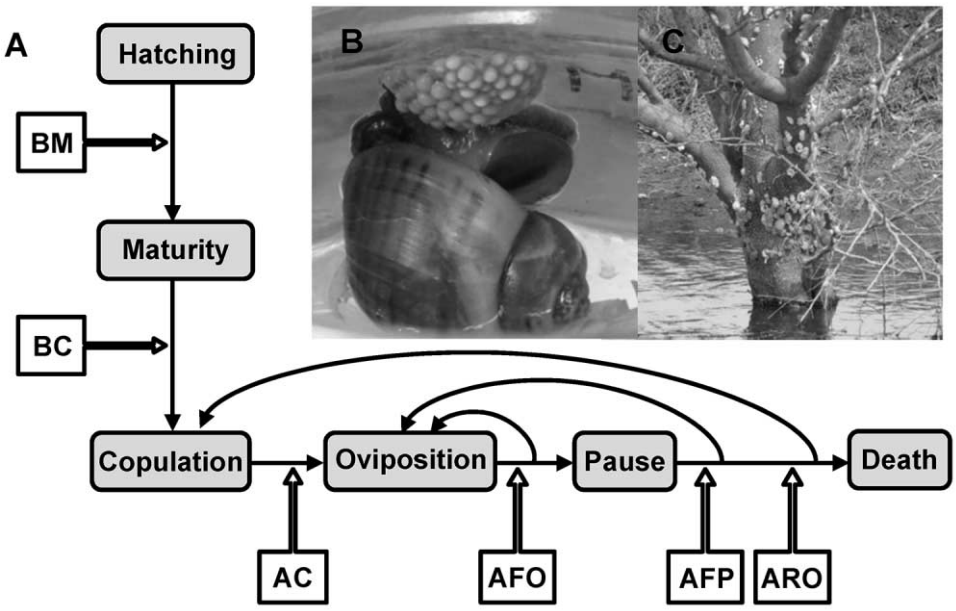

Figure 1. Reproduction in the apple snail *Pomacea canaliculata*. (A) Diagram showing the different phases of the reproductive cycle (gray boxes) and the female groups sampled in this study (white boxes): before maturity (BM), before copulation (BC), after copulation (AC), after first oviposition (AFO), after first pause in oviposition (AFP), and after repeated ovipositions (ARO). (B) Female laying an egg mass in the laboratory. (C) Dozens of egg masses laid on a tree in the Pigüé-Venado channel at the end of the reproductive season.

One of the anatomical features of the female that may allow this remarkable reproductive output is her highly specialized pallial oviduct, which includes the seminal receptaculum, the albumen gland (AG), and the capsule gland (CG), all embedded in a single mass of parenchymal tissue (Hayes *et al.*, 2012). The AG and the CG are deeply intertwined at both cellular and tissue levels (Catalán *et al.*, 2002), forming a glandular complex, referred to as the albumen gland-capsule gland complex (AG-CG) to avoid confusion in the terminology. In particular, the AG synthesizes and secretes perivitelline fluid (PVF), which provides the embryos the nutrients and defenses for development (Heras *et al.*, 1998; Dreon *et al.*, 2013, 2014), while the CG stores large amounts of calcium that later form the eggshell (Runham, 1988). Histological studies of the AG-CG have shown that its parenchymal cells undergo large seasonal variation related to egg production (Catalán *et al.*, 2006).

PVF provides most nutritional embryo requirements in gastropods with an AG (Heras and Pollero, 2002), while yolk in the oocyte is largely reduced and does not supply nutrients (De Jong-Brink *et al.*, 1983). Vitellogenic mechanisms are central to species success in any given habitat, because they reflect species reproductive response to nutrients (Eckelbarger, 1994).

At the biochemical level, some important features of *P. canaliculata* reproduction have also been unveiled. Perivitelline fliud's most abundant perivitellins, namely, PcPV2 and PcOvo, are complex particles exclusively synthesized in the AG-CG and massively accumulated in the eggs, where they account for nearly 70% of egg proteins (Dreon *et al.*, 2002, 2003; Sun *et al.*, 2012). They not only are a source of nutrients but also confer the eggs with nondigestible (antinutritive), antidigestive, and neurotoxic properties that constitute an efficient defense system against vertebrate predators (Heras *et al.*, 2007, 2008; Dreon *et al.*, 2013). Recent findings in other *Pomacea* species showed that the polysaccharide galactogen, the most abundant embryo nutrient, is also nondigestible, adding another defense that at the same time also prevents egg desiccation (Giglio *et al.*, 2016). PcOvo carotenoids additionally provide the eggs with a pink-reddish color, an aposematic warning for potential visual-hunting predators (Heras *et al.*, 2007). These perivitellins and the calcareous eggshell are considered key acquisitions associated with aerial oviposition that could have contributed to the ecological and evolutionary success of the *Pomacea* clade, the most speciose among apple snails (Heras *et al.*, 2007; Hayes *et al.*, 2015).

Ultrastructural and cellular adaptations allowing a high intensity of oocyte release have been reported in the ovary of *P. canaliculata* (Winik and Castro-Vazquez, 2015). In contrast, the biochemical adaptations that allow for the massive production of proteins, polysaccharides, and calcium carbonate of the PVF and the eggshell have not been investigated. To shed light on the metabolism that enables the high reproductive output of *P. canaliculata*, we evaluated the AG-CG in different phases of the reproductive cycle. We focused on the main secretion components: calcium, polysaccharides, soluble proteins, and the most abundant perivitellins, PcOvo and PcPV2; and we also analyzed moisture content and color changes of the complex. In addition, we evaluated whether copulation and/or oviposition could be a stimulus promoting the synthesis of egg secretion components. This study aims to contribute to a better understanding of AG-CG performance and the intrinsic factors limiting the reproductive output of this highly invasive species.

Materials and Methods

The snail and the study area

We collected *Pomacea canaliculata* (Lamarck, 1822) parents from the Pigüé-Venado channel (37°09′59″S, 62°40′28″W) in southwest Buenos Aires Province, Argentina. *Pomacea canaliculata* is the only apple snail inhabiting this area (Martín *et al.*, 2001; Hayes *et al.*, 2012; Seuffert and Martín, 2013); the nearest populations of other *Pomacea* species are located more than 500 km northward. Hayes *et al.* (2012) have confirmed the identity of the snails from the populations used in the present study and in previous ones.

Snail rearing and female sampling

During animal handling and experimentation, we made all efforts to minimize suffering by following *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011). To reduce individual variability, we used full-sibling snails: the snails were born from the same egg mass laid by a virgin laboratory female that was allowed to copulate with only one male (Tamburi and Martín, 2009b). We reared snails from hatching in individual aquaria filled with tap water saturated with calcium carbonate, under controlled conditions of temperature and photoperiod (25 °C ± 2 °C and light : dark cycle of 14 h : 10 h), and we fed them fresh lettuce *ad libitum*. When the snails reached two months of age, we sexed them under a stereomicroscope; those with the testis in the apex, visible through the translucent shell were considered males, and the remaining were considered females (Tamburi and Martín, 2009b). We transferred females and males to individual 3-L aquaria maintained under the conditions stated above.

When the cohort reached an age of four months, we randomly chose five females and assigned them to a reference group; we used these females to check that the snails were ready to copulate. To check this, twice a week we put a male into the aquarium of each female. Once four out of five females from this reference group copulated, we considered that the cohort had reached sexual maturity. Three weeks after sexual maturity, we assigned experimental females to six groups to be sampled along different phases of the reproductive cycle (Fig. 1A).

We randomly assigned the females to groups as follows (Fig. 1A). The first day, we sampled six females (mature vir-

gins) before copulation (BC group). Thereafter, to each of the remaining experimental females we daily offered for 6 h a randomly selected consort male with which to copulate; each couple was observed and checked every 20 min from 08:00 to 20:00 hours. We allowed females to copulate only once; to be considered a successful copulation, the intromission of the penial sheath into the female pallial cavity should last a minimum of three hours (Burela and Martín, 2011). We sampled 6 females less than 1 h after their first copulation had finished (after copulation, AC group), and we sampled 6 females less than 12 h after their first oviposition (AFO group). We sampled 6 females after the first pause in oviposition (AFP group, *i.e.*, after 24–36 h had passed without a new oviposition). We allowed a group of 4 females to freely oviposit egg masses up to the end of the sampling period (17 ± 6 d, on average) before sampling them (after repeated ovipositions, ARO group). At the end of the experimental period (38 d), 5 females had shown no copulatory activity, even after 20 opportunities with different consort males. We considered these females as still sexually immature (before maturity, BM group).

Determination of female and egg biomass

For each female, we recorded the number of oviposition events, obtained the wet weight of each egg mass with a digital scale, and calculated the total dry weight of all the deposited egg masses from data of Heras *et al.* (1998). We measured the total shell length (from the apex to the opposite aperture rim) with a vernier caliper to the nearest tenth of millimeter and then weighed whole individual (total weight). Then we removed the shell, dissected the pallial oviduct, and weighed the remaining soft body parts separately.

Preparation of the AG-CG samples

We weighed the AG-CG and stored nearly 1 mg of parenchymal tissue in RNA*later* (Ambion, Foster City, CA), following the manufacturer's specifications for RNA extraction. We stored the remaining tissue of the AG-CG at −70 °C until use for biochemical analysis. We analyzed each AG-CG individually. We determined the moisture content of the AG-CG by drying the tissues at 130 °C for 19 h, except otherwise stated, and we analyzed the samples from the 6 groups.

We homogenized the samples in ice-cold 20 mmol L^{-1} Tris-HCl, pH 7.5, containing a general cocktail of protease inhibitors (cat. no. P2714, Sigma-Aldrich, St. Louis, MO), on a Potter-Elvehjem-type homogenizer and using a buffer : sample ratio of 9 : 1 v/w. We sequentially centrifuged the crude homogenate at 10,000 × *g* for 20 min and at 100,000 × *g* for 50 min and then discarded the pellets.

Calcium quantification

We determined soluble calcium colorimetrically (Ca-Color, Wiener Laboratorios S.A.I.C., Rosario, Argentina) on AG-CG cytosol, using an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA). For total calcium determination, we previously performed an acidic digestion of the organ. Briefly, we homogenized each sample in ultrapure water 1 : 3 w/v and digested each overnight at 4 °C with concentrated HNO_3 2 : 1 v/v acid : homogenate. We then neutralized it with NaOH 12 mol L^{-1} and centrifuged for 10 min at 13,600 × *g*. We measured the solubilized calcium colorimetrically as described above. We calculated nonsoluble calcium in the AG-CG of each female as the difference between total and soluble calcium.

Total polysaccharide quantification

We precipitated polysaccharides by the method described by Van Handel (1965) and then quantified them colorimetrically by the anthrone-sulphuric acid method. Briefly, we added 1.5 mL of anthrone 0.2% in H_2SO_4 84% to 0.25 mL of sample solution. We measured absorbance at 620 nm in an Agilent 8453 UV-visible spectrophotometer using galactose (Sigma-Aldrich) as standard.

Soluble protein quantification

We determined total soluble protein concentration colorimetrically (Lowry *et al.*, 1951) on the cytosolic fraction of the AG-CG in an Agilent 8453 UV-visible spectrophotometer. We used bovine serum albumin (BSA) as standard (Sigma-Aldrich).

PcOvo and PcPV2 gene expression

We quantified gene expression of the main perivitellins PcOvo and PcPV2 by quantitative polymerase chain reaction (qPCR) on the BC, AC, AFO, and AFP groups. We extracted total RNA from the AG-CG samples by using Trizol reagent (Invitrogen, Carlsbad, CA). We designed specific primers for PcOvo and PcPV2 (Table 1) from the nucleotide sequences (Sun *et al.*, 2012), using the software Primer 3 (Koressaar and Remm, 2007; Untergasser *et al.*, 2012) and Primer-BLAST (Ye *et al.*, 2012). Then we analyzed them using NetPrimer (Premier Biosoft, Palo Alto, CA). We confirmed the specificity of each primer pair by analyzing the amplification products by electrophoresis and their melting curves. We used glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-actin (*ACTB*) as internal controls for expression normalization according to a reference gene validation study in the *P. canaliculata* AG-CG (Cadierno *et al.*, 2018). We conducted qPCR following the method described by Cadierno *et al.* (2018). In short, we performed qPCR in a Stratagene Mx3005P qPCR instrument (Agilent), using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Cycling conditions were 1 min at 95 °C, 40 cycles of 10 s at 95 °C, 30 s at the specific annealing temperature (see Table 1), and 30 s at 60 °C, with 1-min hold at 95 °C after the cycling

Table 1

Primer sequences for perivitellins and reference genes of Pomacea canaliculata

Gene	Forward primer	Reverse primer	Amplicon length (bp)	Annealing temperature (°C)	Amplification efficiency (%)
PcOvo[a]	GTGGCTACATCCCTGTTGCT	CCCTGAACGAGTGCTTGACT	154	55	96.7
PcPV2[b]	GGTGTTGGGCAAGTTGATGT	ACGAGAAGAGAAACGGGTGT	183	53	96.6
ACTB[c]	TCACCATTGGCAACGAGCGAT	TCTCGTGAATACCAGCCGACT	87	56	93.7
GAPDH[c]	CAACCTCAAAACCGATGCCA	GACAAAGCGATTAGTCAGTGGA	184	55	96.3

[a] Primer design based on PcOvo subunit 1 (accession no. JQ818217).
[b] Primer design based on PcPV2 minor subunit (accession no. JX155862).
[c] Reference genes selected from Cadierno *et al.* (2018).

step. We performed a melting curve analysis from 55 °C to 95 °C. We measured each gene in the same run, including the standard curve and the complete set of samples. We made a standard curve using serial dilutions (1/5, 1/10, 1/50, 1/100, and 1/500) of females' cDNA from a pool made from all treatments. We diluted individual female's cDNA samples 1/10 for analysis. We performed all reactions as technical triplicates. Finally, we also included a no-template control (NTC) and a no-retrotranscription control (NRT) on each plate. We calculated the primer pair efficiency from the slope of the linear relation between the Ct (cycle threshold) values and the standard curve points.

Quantification of PcOvo and PcPV2 levels

We quantified total perivitellin levels of the AG-CG by the enzyme-linked immunosorbent assay (ELISA). The procedure follows that described by Dreon *et al.* (2003), with slight modifications. We prepared the standard curve using PcOvo and PcPV2 purified from the AG-CG. We performed blocking with 1% BSA (Sigma-Aldrich). We diluted the anti-PcOvo and anti-PcPV2 rabbit serums in phosphate-buffered saline (PBS)-Tween (1/5000 and 1/20,000, respectively) containing 0.5% BSA. We diluted goat anti-rabbit immunoglobulin G (IgG) horseradish peroxidase conjugate (Bio-Rad Laboratories) 1/250 and 1/1000 for PcOvo and PcPV2, respectively, in the abovementioned antibody solution. We measured absorbance at 405 nm in a microplate reader (DTX 880 Multimode Detector, Beckman Coulter, Brea, CA).

Determination of AG-CG color

To evaluate AG-CG color differences, we registered the absorbance spectrum of its cytosol. We recorded UV-visible spectrum in 20 mmol L^{-1} Tris buffer, pH 7.5, at room temperature in an Agilent 8453 UV-visible spectrophotometer. Its visible region absorbance profile mainly corresponds to PcOvo carotenoprotein, which has a series of associated chromogens (Dreon *et al.*, 2004). Because this perivitellin exhibits 3 absorbance maxima, and the most intense one occurs at 509 nm (Cheesman, 1958), we calculated an absorbance ratio of 509/280 nm as indicative of PcOvo relative contribution to the visible spectrum.

Statistical analysis

We compared all the variables using one-way analyses of variance (ANOVAs) and Bonferroni *post hoc* tests to locate differences between groups, for either original or transformed variables. In the case where we rejected Levene's test for homogeneity of variances of a given variable ($P < 0.05$) and where there was no effective transformation, we performed Kruskal-Wallis tests followed by Dunn's tests. We estimated the association among selected variables using the Pearson's correlation coefficient.

Results

Biomass of females, AG-CG, and egg masses

AG-CG dry weight showed a significant variation between groups ($F_{5,26} = 10.976$, $P < 0.001$), reaching a minimum in the ARO group (Fig. 2A); while total shell length, total weight, and soft parts weight (excluding the pallial oviduct) did not vary between female groups ($P > 0.097$ in all cases; Table 2). As a consequence, the ratio between the AG-CG and soft parts exhibited differences between female groups ($F_{5,26} = 16.095$, $P < 0.001$). There was a negative correlation between AG-CG weight and total egg mass deposited by each female on a dry weight basis ($r = -0.806$, $P < 0.001$; $n = 16$; Fig. 2B). Egg mass wet weight decreased with the successive oviposition events (Fig. 2C). The number of egg masses per female for the groups AFO, AFP, and ARO were 1, 3.3 ± 1.03 and 8.25 ± 2.36 (mean ± SD), respectively.

Main AG-CG secretion compounds

While AG-CG moisture content showed a significant increase in the AFP and ARO groups ($F_{5,26} = 20.568$, $P < 0.001$; Fig. 3A), the amount of soluble proteins and polysaccharides significantly decreased only in the ARO group ($F_{5,27} = 6.532$, $P < 0.001$ and $F_{5,22} = 7.270$, $P < 0.001$, respectively; Fig. 3B, C). Total and nonsoluble calcium showed

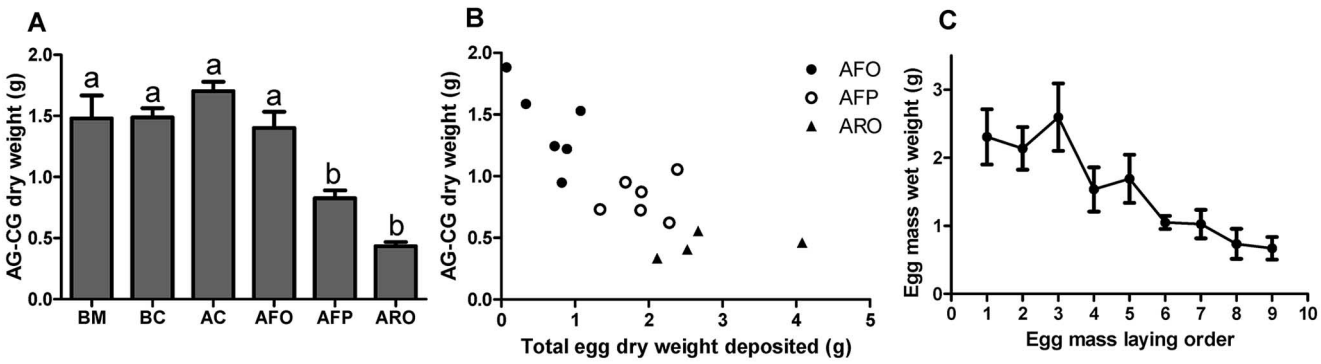

Figure 2. Weight changes in *Pomacea canaliculata* albumen gland-capsule gland complex (AG-CG) and egg mass along the reproductive cycle. (A) AG-CG biomass (total dry weight mean ± SE). (B) Correlation between AG-CG biomass and total egg dry biomass deposited. (C) Egg mass along successive ovipositions (wet weight, mean ± SE). Sampling points: before maturity (BM), before copulation (BC), after copulation (AC), after first oviposition (AFO), after first pause in oviposition (AFP), and after repeated ovipositions (ARO). Different lowercase letters above bars indicate significant differences between groups ($P < 0.05$).

stable levels along the reproductive phases analyzed (Kruskal-Wallis tests: $X^2 = 4.815$, $P = 0.439$ and $X^2_5 = 4.954$, $P = 0.422$, respectively; $n = 33$). Soluble calcium levels (23.3% ± 11.7% of total calcium for the BC group) varied ($F_{5, 27} = 2.574$, $P = 0.050$), showing differences between the BM and AFP groups but not between females with different reproductive outputs (Fig. 3D).

Stored levels and gene expression of PcOvo and PcPV2

The stored level of the perivitellins PcOvo and PcPV2 within the AG-CG decreased along the phases of the reproductive cycle ($F_{5, 27} = 5.972$, $P < 0.001$ and $F_{5, 27} = 6.661$, $P < 0.001$, respectively), with the ARO group showing the lowest level (Fig. 4A, B). This was especially evident in PcOvo, which decreased to half the protein level present in both virgin females, either immature or mature (BM and BC). Gene expression levels of PcOvo and PcPV2, quantified as mRNA, showed a significant overexpression in the AFP group ($X^2_3 = 10.33$, $P = 0.0159$ and $X^2_3 = 10.34$, $P = 0.0157$, respectively), which nearly duplicated and triplicated, respectively, the basal level present in the BC, AC, and AFO groups (Fig. 4C, D). The qPCR efficiency value had a satisfactory result for all primer pairs (Table 1). Each amplification product showed a single band with the expected length in agarose gel electrophoresis, and melting curve analysis further confirmed primer specificity (not shown).

Absorbance spectra of AG-CG cytosol

AG-CG cytosol spectra showed similar profiles regardless of the female group evaluated (Fig. 5A). When spectra were normalized to 280-nm absorbance, differences in the ratio 509/280 nm were observed between BM and ARO females (Kruskal-Wallis test, $X^2_5 = 16.272$, $P = 0.006$; Fig. 5B). Macroscopically, the AG-CG appearance of ARO females was clearly less turgid and its color less intense than that of the other groups.

Discussion

The present work provides a biochemical view of the remarkable reproductive output of *Pomacea canaliculata* during the reproductive cycle, focusing on AG-CG performance under low, intermediate, and high reproductive outputs. The gland shows high biosynthetic activity, with an efficient restoration process for proteins and polysaccharides at low and intermediate reproductive outputs. However, females cannot

Table 2

Length and wet weight from females along the reproductive cycle of Pomacea canaliculata

Variable	BM	BC	AC	AFO	AFP	ARO
Total shell length (mm)	46.1 ± 0.4	42.8 ± 0.3	45.5 ± 0.3	45.4 ± 0.4	44.1 ± 0.4	45.7 ± 0.3
Total weight (g)	22.7 ± 0.8	17.9 ± 0.4	19.7 ± 0.4	20.9 ± 0.4	18.4 ± 0.5	21.3 ± 0.3
Soft parts weight (g)	8.9 ± 0.2	7.3 ± 0.1	7.6 ± 0.2	7.9 ± 0.2	7.9 ± 0.2	8.1 ± 0.2

Sampling points: before maturity (BM), before copulation (BC), after copulation (AC), after first oviposition (AFO), after first pause in oviposition (AFP), and after repeated ovipositions (ARO). Values indicate mean ± SE. Soft parts weight does not include the pallial oviduct (albumen gland-capsule gland complex plus seminal receptaculum).

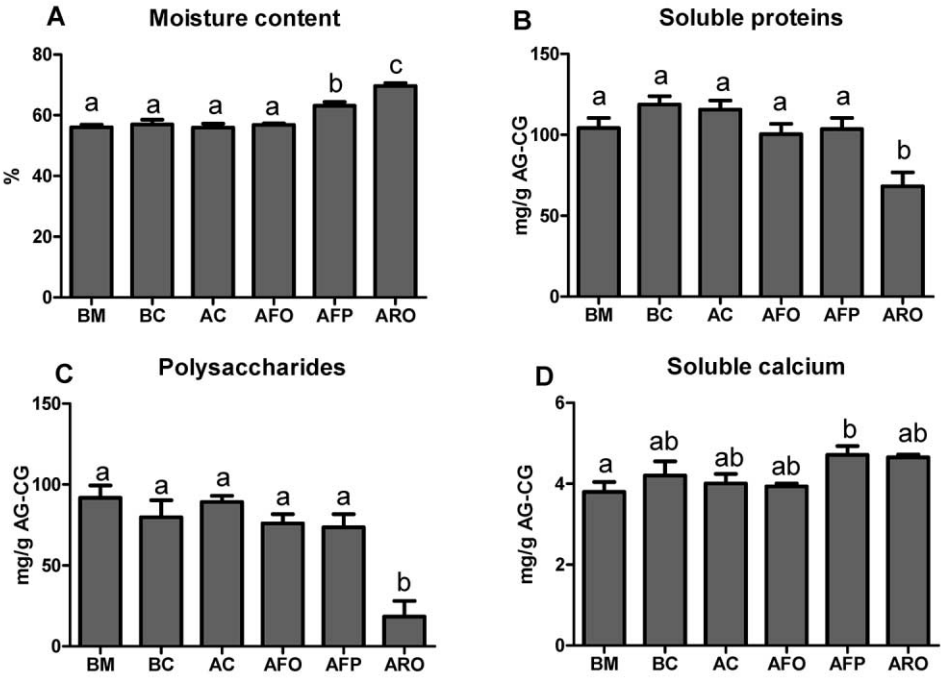

Figure 3. Changes in the biochemical composition of *Pomacea canaliculata* albumen gland-capsule gland complex (AG-CG) along the reproductive cycle (mean ± SE). (A) AG-CG moisture content. (B) Soluble proteins. (C) Polysaccharides. (D) Soluble calcium. Sampling points: before maturity (BM), before copulation (BC), after copulation (AC), after first oviposition (AFO), after first pause in oviposition (AFP), and after repeated ovipositions (ARO). Different lowercase letters above bars indicate significant differences between groups ($P < 0.05$).

cope with a high reproductive effort, and gland biosynthesis seems to be a key factor limiting the reproductive potential. Despite the genetic and environmental homogeneity of the females, there was a great variability in their onset of reproductive activity (from 1 to more than 38 days after the experiment started). This asynchrony was previously observed for laboratory-reared *P. canaliculata* females, in which age at maturity varied up to 150 days within the same population (Martín and Estebenet, 2002). This variability in our study cannot be ascribed to the main secretion components in the AG-CG because the immature and mature females (BM and BC groups) showed no differences in any of the variables analyzed.

Mechanisms of egg production are phylogenetically constrained and therefore intrinsic to the species and not affected by environmental factors (Eckelbarger and Watling, 1995). In this regard, recent reports indicate that apple snails and probably other gastropods have evolved an alternative vitellogenic mechanism whereby perivitellins, unlike vitellins of other invertebrates, are synthesized as already functional in the albumen gland (Cadierno *et al.*, 2017). This provides snails with a more efficient and faster mechanism for food conversion into egg production (Cadierno *et al.*, 2017). In this study, the decrease of AG-CG polysaccharides, soluble proteins, and the most abundant perivitellins in the ARO group reflects the massive transfer of perivitelline fluid compounds to the eggs, providing embryos with nutrients and defenses during development. Additional evidence of the large female capacity for egg production is the total egg dry weight deposited by the ARO group, which almost doubled AG-CG dry weight of the BC group, indicating that the AG-CG has a fast restoration process. Further, at the gene expression level, perivitellins from females with an intermediate reproductive output (AFP group) almost doubled (PcOvo) or tripled (PcPV2) their mRNA expression.

As a whole, these results highlight the high biosynthetic activity of the organ. This agrees with previous studies on AG perivitellin synthesis (Dreon *et al.*, 2002, 2003) and its storage in granules of secretory cells during the reproductive period (Catalán *et al.*, 2006). A similar restoration period after the release of PVF during oviposition was observed for the AG of the pulmonate snail *Lymnaea stagnalis* (Wijsman and Wijck-Batenburg, 1987). In *P. canaliculata* and other freshwater snail species, this synthesis and release during multiple oviposition events seem to be under neuroendocrine control (Mukai *et al.*, 2004; Catalán *et al.*, 2006). It is worth recalling that multiple spawnings are characteristic of an opportunistic reproductive strategy, commonly found in species that inhabit adverse or unpredictable environments (Chiarello-Sosa *et al.*, 2016).

Our findings suggest that perivitellin expression would be regulated at the transcriptional level, though more studies are needed to determine whether there are other regulatory steps

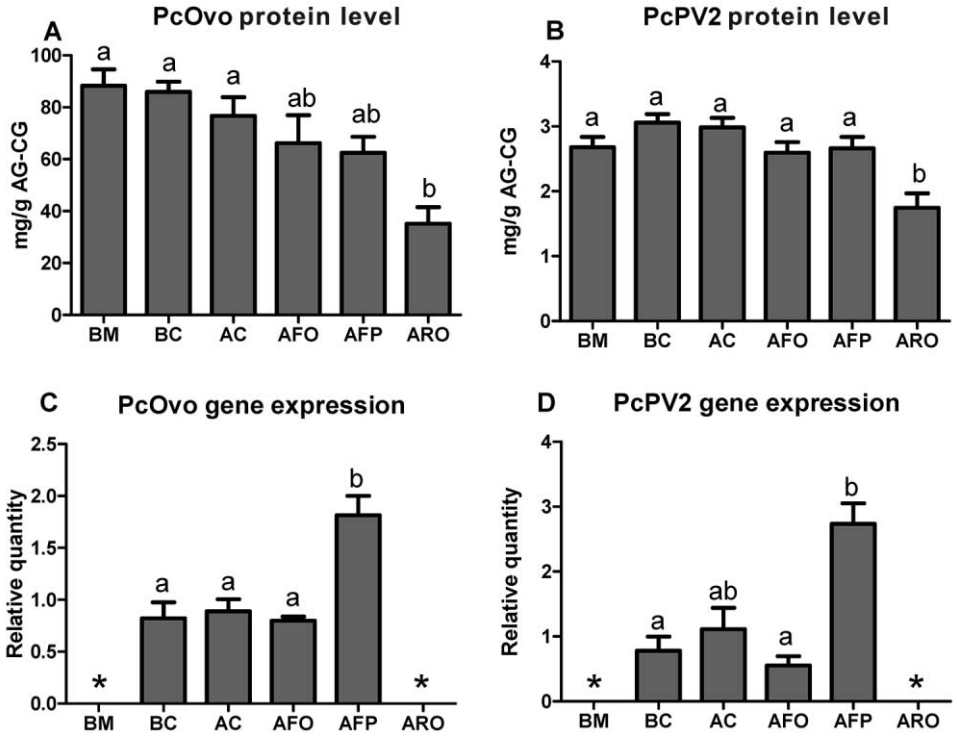

Figure 4. Changes in *Pomacea canaliculata* albumen gland-capsule gland complex (AG-CG) level and expression (mean ± SE) of the most abundant perivitellins along the reproductive cycle. (A) PcOvo protein level. (B) PcPV2 protein level. (C) PcOvo mRNA expression. (D) PcPV2 mRNA expression. Sampling points: before maturity (BM), before copulation (BC), after copulation (AC), after first oviposition (AFO), after first pause in oviposition (AFP), and after repeated ovipositions (ARO). Different lowercase letters above bars indicate significant differences between groups ($P < 0.05$).

at this reproductive stage and to determine whether this restoration process is important for the other secretion components. In this regard, at least polysaccharides follow the same fluctuation as perivitellins, probably a reflection of the synchronized synthesis and storage of polysaccharides by the secretory cells within the same granules that contain the perivitellins (Catalán *et al.*, 2006). In agreement with the levels of perivitellin gene expression, the amount of these proteins accumulated in the AG-CG did not differ between the AFO (one egg mass) and AFP (about three egg masses) groups. This indicates an efficient biosynthetic restoration process at low and intermediate reproductive outputs. However, after a high reproductive effort, restoration is not enough to maintain the initial reproductive potential, which in any case is remarkable, considering

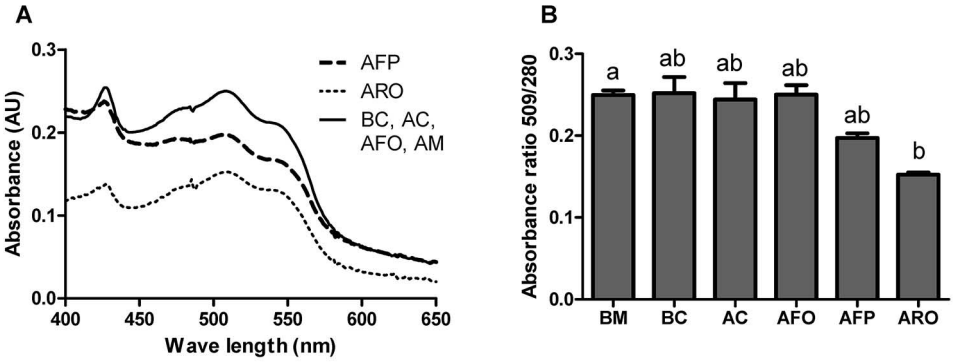

Figure 5. Changes in the visible spectra of *Pomacea canaliculata* albumen gland-capsule gland complex (AG-CG) cytosol along the reproductive cycle. (A) Absorbance spectra. (B) Relative contribution of PcOvo perivitellin to the absorbance spectra (mean ± SE). Sampling points: before maturity (BM), before copulation (BC), after copulation (AC), after first oviposition (AFO), after first pause in oviposition (AFP), and after repeated ovipositions (ARO). Different lowercase letters above bars indicate significant differences between groups ($P < 0.05$).

that females from the ARO group deposited ~1400 ± 400 eggs (estimated from Tamburi and Martín, 2011). The same profile is paralleled by soluble proteins and polysaccharides, which decreased between the BC and ARO groups, suggesting that the biochemical secretion components limit the reproductive output in *P. canaliculata*.

The total amount of proteins and polysaccharides transferred to eggs was estimated by combining *P. canaliculata* egg data (Heras *et al.*, 1998) with the total weight of eggs deposited, considering that egg quality does not vary along several oviposition events (Frassa, 2011; Tamburi and Martín, 2011). The initial amount of proteins and polysaccharides stored in the AG-CG of the BC group was 2.1 and 8.2 times lower, respectively, than the amount transferred to eggs by the ARO group. This further supports the notion that there is an efficient and quick restoration process in the AG-CG that enables females to supply eggs with the appropriate nutritional quality and quantity.

In contrast with the behavior of organic molecules, total and nonsoluble calcium showed constant levels in the AG-CG regardless of the phase of the reproductive cycle or the reproductive effort. Calcium reserves preferentially accumulate in females during the pre-reproductive period, as reflected by a 30% higher ash content of young females' bodies relative to young males' (Estebenet and Martín, 2003). Ultrastructural analysis showed that calcium accumulates as intra- and intercellular calcareous spherules (Catalán *et al.*, 2002, 2006). Another difference between proteins and polysaccharides was that the total calcium stored in the AG-CG of the BC group was 1.5 times higher than the total calcium transferred to eggs by the ARO group (calculated from Giglio *et al.*, 2016). This indicates that an efficient restoration of calcium reserves is taking place, because, otherwise, AG-CG calcium would be almost completely depleted. This agrees with the histology of the AG-CG, where large calcium deposits that infiltrate the cytoplasm and accumulate in the extracellular space of the basal labyrinth during the reproductive period were observed (Catalán *et al.*, 2006).

The restoration mechanism of AG-CG calcium during the reproductive season is currently unknown, but it probably involves either a bypass of its circulation and deposition into the shell and/or a mobilization from the shell to the AG-CG. These alternative routes are supported by the fact that shells of females that oviposit actively until their natural death are 30% lighter than those of males of the same size, whereas the shell weight of young mature snails without reproductive activity shows no intersexual differences (Estebenet and Martín, 2003). These intersexual shell differences were evident despite the snails being reared in $CaCO_3$-saturated water and with *ad libitum* lettuce, indicating that they were independent of environmental calcium. Soluble calcium levels remained almost constant along the reproductive period, maintaining the storage levels of the AG-CG at least during the 38 days of the experiment. Catalán *et al.* (2007) reported a drop in intra- and intercellular calcium storage in the AG-CG only after 180 days of reproductive activity.

Several pieces of information support *P. canaliculata* as a fast egg producer largely dependent on food availability. First, the absence of differences in soft part weight (excluding the pallial oviduct) between groups indicates a restoration of AG-CG secretion compounds supported by the continuous nutrient input from the food ingested *ad libitum* and calcium mobilized, either from the shell or directly from the environment (see above). Second, *P. canaliculata* reproductive output largely depends on food availability (Estoy *et al.*, 2002a), and, as a consequence, egg laying declines during fasting periods, with a rapid recovery after the food supply is restored (Tamburi and Martín, 2016). Finally, females have food ingestion rates 50% higher than males of the same size (Tamburi and Martín, 2009a), an adaptation that probably fulfills AG-CG requirements during the reproductive season.

The number and quality of eggs may be affected by environmental factors such as food availability and quality, stress in physical factors, or toxicity (Eckelbarger, 1994). AG protein biosynthesis is a process with high nitrogen demand. Our study supports the notion that this process limits egg production, which is in agreement with previous reports indicating that the nitrogen content of food was positively correlated with egg production (Qiu and Kwong, 2009). Under our experimental setting, females with *ad libitum* food supply became unable to cope with this high demand after repeated ovipositions. A reduction in the number but not quality of eggs after multiple spawnings has also been observed in other species (Wootton and Fletcher, 2009). In our study, females under high demand of biosynthesis showed macroscopic changes in the general aspect of the gland and its coloration. In the ARO group, the AG-CG color was pale pink and presented greater flaccidity, probably because of the increase in water content, while it was bright pink-reddish and turgid in all the other groups (not shown). Moreover, the 509/280-nm absorbance ratio decreased between the BM and ARO groups. Considering the remarkable diversity in AG-CG color intensity commonly observed in females sampled in the field (MPC and PRM, pers. obs.), the decrease in the 509/280-nm absorbance ratio in the ARO group provides a useful baseline to determine whether this parameter could provide information on females' past reproductive output.

In summary, our results show that the AG-CG of a fast egg producer restores the main secretion compounds during a period of high reproductive output with different efficiencies, maintaining its calcium reserves but not organic compounds, which therefore seems to limit a constantly high reproductive output. In this regard, the metabolic parameters determined are useful to evaluate the reproductive activity of the organ and factors affecting fecundity in *P. canaliculata* and other related species. More comparative work is needed because there is little information on AG-CG perivitellin synthesis and dynamics in other gastropods. More work is also needed

comparing AG-CG dynamics of *P. canaliculata* from temperate regions (this study) with that in tropical or subtropical regions where the snails lay eggs continuously throughout the year. The present work identified AG-CG biosynthesis as a potential target for developing control strategies aimed at reducing apple snail invasiveness.

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