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Effects of absolute fasting on reproduction and survival of the invasive apple snail *Pomacea canaliculata* in its native range

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Received on 29 August 2015; accepted on 26 November 2015

Abstract

A South American freshwater gastropod, the apple snail *Pomacea canaliculata*, has become a driver of ecosystemic changes in wetlands and an important rice pest after its introduction to various parts of the world, mainly Asia. The objective of this study was to study the effect of an abrupt interruption in food availability in the short term (up to 4 weeks) and long term (up to 8 months) on survival and reproductive activity. The main results indicate that short-term fasting mainly affects the survival of males, but only when they are raised together with females, probably due to a greater mate-searching activity that increases mortality in the individuals with lower reserves. The number of copulating snails or egg-laying females shows an abrupt drop when fasting and a rapid recovery after the food supply is restored. The strategy of discontinuing reproductive activity prioritizes energy conservation for the survival of the females. Interpopulation variation in resistance to starvation was observed in adults, which can be explained to some extent by the food availability that they experienced in their natural environment. No interpopulational differences in survival were seen in hatchlings. The mean maximum values of survival under starvation were 52.6 days in hatchlings and the 3.3% of adults survive over than 200 days, which may be a relevant trait in dispersal and establishment in new habitats.

Invasive species are considered one of the major threats to biodiversity and also the source of huge economic losses (Gurevitch and Padilla 2004; Molnar et al. 2008; Hulme et al. 2009). Many species of freshwater snails have the potential to damage ecosystem functioning or structure (Cowie et al. 2009; Strayer 2010), but only one has been listed among the 100 world's worst invasive species by the International Union for Conservation of Nature (Lowe et al. 2000). The apple snail *Pomacea canaliculata* (Lamarck 1822) is native of South America that has been introduced in various parts of the world causing serious damage in aquatic crops and natural wetlands (Horgan et al. 2014; Hayes et al. 2015). In Asia, it causes economic losses of several billions of dollars (Joshi and Sebastián 2006; Nghiem et al. 2013) and it is capable of generating notable ecosystem changes in natural wetlands (Carlsson et al. 2004). In addition, it has been indicated as one of the predominant intermediate hosts of

Angiostrongylus cantonensis, the causal agent of eosinophilic meningitis, an emerging parasitic disease in China (Lv et al. 2009 2011). Recent introductions have been recorded in the Conchalí River in Chile (Jackson and Jackson 2009) and in the delta of Ebro River in Spain (Soriano et al. 2009), where it has raised high concern about potential agricultural and ecosystemic impacts (EFSA 2013, 2014).

The ability of *P. canaliculata* to become established in new habitats when introduced may be attributed to some key life-history traits (e.g., the capacity of females to preserve sperm for several months and to lay thousands of viable eggs over a reproductive season; Estebenet and Martín 2002; Burela and Martín 2011) and physiological capacities (e.g., a dual respiratory system consisting of a gill and a lung; Andrews 1965). Moreover, they can survive in dry conditions for up to 13 months and buried in wet mud for up to 29 months (Fan et al. 2000; Yusa et al. 2006). The ability to survive

during these long periods out of water is based on behavioral changes, such as inactivity, and physiological changes, such as a decrease in the metabolic rate and changing to anaerobic metabolism (Hayes et al. 2015). Lach et al. (2000) found that *P. canaliculata* may be able to survive through lengthy periods of low food availability in water but also cast doubts about whether starved snails would mature and reproduce. Further studies showed that *P. canaliculata* is capable of maturing, mating, and laying viable eggs under chronic restrictions of up to 80% food availability since hatching while maintaining a null pre-reproductive mortality (Tamburi and Martín 2009a, 2011). However, the effect of periods of absolute fasting on reproductive activity and survival when the snails remain in water is still unknown. The ability to resist prolonged fasting in water may be a key factor in the accidental or intentional dispersal of this species and in its chances of becoming established in habitats managed by humankind, where food availability varies in an unnatural or unpredictable way. Survival and fertility represent 2 fundamental parameters in population dynamics which are also necessary to develop predictive models of population growth of an invasive species such as *P. canaliculata* in new environments or under alternative scenarios (e.g., EFSA 2013, 2014).

In its native range *P. canaliculata* shows great interpopulational variation in its life-history traits, partly attributable to genetic variation and partly to phenotypic plasticity (Martín and Estebenet 2002; Estebenet and Martín 2003). Some of these life-history traits show differences between the sexes in the way they react to a food availability gradient (Estoy et al. 2002; Tamburi and Martín 2009a). On the other hand, maternal effects were found in the survival of starved hatchlings as the offspring of mothers that lived under low food availability resisted longer (Tamburi and Martín 2011). These facts suggest that there may also be ecophenotypic differences in the ability to resist absolute fasting in the adults and hatchlings between populations with different food availabilities.

The objective of this study was to evaluate the effect of fasting on *P. canaliculata* when the snails remained in water. The effect of short-term fasting on reproductive activity, survival, and growth was studied, and also the effect of long-term fasting (starvation) on the survival of adults and hatchlings from populations with different food availabilities.

Materials and Methods

The snail and the study area

All the sampling and collection of snails for the experiments described below took place in the Encadenadas del Oeste, an closed basin in Southern Pampas (Buenos Aires, 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 northwards. The identity of the snails from the populations used in the present study and in previous ones has been confirmed by Hayes et al. (2012).

The effects of short-term fasting in adults

The objective of the short-term fasting experiment was to determine the effect of absolute fasting of short duration (1–3 weeks) on survival, reproduction and growth of adult snails in an aquatic environment. Adult snails were collected in a shallow watercourse that connects the Curamalal Grande stream with the Cochicó Chico stream (36°59'40.89"S; 62°12'6.49"W) in February 2008. Snails were sexed,

forty couples were selected at random and each couple was kept individually in a 3-l container. The initial total length (SL_i , mm) was measured with a caliper (± 0.05 mm) and the live weight (LW_i , g) was obtained with a digital scale (± 0.001 g) after the snail was allowed to crawl for 5 min on a plastic tray to drain the water from the pallial cavity. Snails used in the study measure initially 42.45 ± 5.32 in the case of males and 44.07 ± 2.39 in the case of females (mean \pm SD). The couples were maintained under controlled conditions (temperature: $25 \pm 1^\circ\text{C}$; photoperiod: 14:10 h light:dark). The aquariums were cleaned, and the water was replaced weekly with tap water and calcium carbonate added to saturation.

Three treatments and a control were set up with 10 couples in each. The first week was of acclimatization with food *ad libitum* for all treatments. An absolute fasting period of 1, 2, or 3 weeks was applied to the different treatments after the acclimatization week. At the end of each fasting period, food was restored up to the fourth week of the experiment. The control had food *ad libitum* all the time. The water was renewed twice a week, on Fridays and Tuesdays if food was available to the snails and only on Fridays during fasting.

Every day, the copulations were recorded at 9.00 am and any egg masses laid in the aquarium were collected. This procedure allowed to register most copulations since under laboratory conditions the copulatory activity of *P. canaliculata* is more frequent in the morning (Albrecht et al. 1996) and the copulations last for 12.15 h on average (Burela and Martín 2011). The egg masses were disaggregated in 50 ml of sodium hypochlorite solution (55 g/L) and the eggs counted.

Survival and growth in live weight (LW, g) of the experimental specimens were recorded during the four-week experiment. The Kaplan–Meier method was applied to test the equality of survival distribution between the treatments using Tarone–Ware tests (Tarone and Ware 1977). The same analysis was used to compare the survival distribution of males and females.

The corrected copulatory activity was calculated as the number of copulation events recorded in respect of the total number of potential events (i.e., the number of couples that are still alive at the end of the week multiplied by the 5 moments of weekly observation). The egg-laying activity was calculated as the number of females that laid eggs during the week with respect of the number of aquariums that ended the week with a live female. Both variables were analyzed for the values of the last week of fasting in each treatment, and a mean value was used for the 3 weeks in the control. A chi-square general test was performed with the results expected under homogeneity and chi-square tests were individually performed to compare each treatment with the control at the corresponding week of fasting.

To estimate growth and somatic state 2 variables were used. The variable relative weight change during the entire fasting period was calculated as $(LW_{\text{after}} - LW_{\text{before}}) / LW_{\text{before}}$, while the condition factor (CF, g/mm) was calculated as the log-transformed ratio between the initial live weight and the initial length ($CF = \log_{10}(LW_i / SL_i) + 1$).

Levene and Kolmogorov–Smirnov tests were used to analyze the homogeneity of variances and normality assumptions and to determine whether to perform parametric tests (ANOVA (analysis of variance), *t*-test) or nonparametric tests (Kruskal–Wallis, Mann–Whitney *U* test). The relative weight change during fasting was tested with a two-way ANOVA and, within each sex, it was tested with *t*-tests to see if the overall average differed from zero (i.e., no change in weight). To investigate the cause of death of the snails, irrespective of the fasting period suffered, the condition factor of the

animals that died or survived until the end of the experiment was compared for each sex by using *t*-tests.

The effects of long-term fasting in adults

The objective of the long-term fasting experiment was to determine the interpopulational and intersexual variation in survival under long-term absolute fasting (starvation). The snails were collected at 3 sites in the Guaminí stream catchment area (Buenos Aires province, Argentina) in February 2014: site A ($37^{\circ}20'48.80''S$ $62^{\circ}25'16.89''O$) and B ($37^{\circ}23'51.76''S$ $62^{\circ}23'38.91''O$) are on the Guaminí stream, whereas point C ($37^{\circ}17'21.73''S$ $62^{\circ}20'54.91''O$) belongs to Corto stream, an affluent of the Guaminí stream. These 3 sites were selected for their proximity (less than 10 km between them), connectivity (absence of barriers to dispersal), and their different food availabilities. Snails were searched for by 2 people in the submersed vegetation and mud while wading upstream and picked by hand (Martín et al. 2001). The population relative abundance was estimated by calculating the number of specimens collected in unit time by each operator (60, 60, and 46 min of searching time for A, B, and C, respectively).

The actual food availability that *P. canaliculata* experiences in the field cannot be confidently estimated from aquatic vegetation abundance, due to the different palatabilities of aquatic plants and the diverse feeding mechanisms (Wong et al. 2010; Morrison and Hay 2011; Saveanu and Martín 2015). Hence, somatic indices are a practical approach to estimate the actual trophic availability that snails experience in the field (Tamburi and Martín 2009a, 2012). The difference in shell length at maturity between sexes is negatively related to trophic availability (Tamburi and Martín 2009a). The total shell length of the male and female of each mating couple at the 3 sites was recorded ($n = 28, 10,$ and 15 couples for A, B, and C, respectively), and for each sex the first quartile was calculated to estimate minimum size at maturity.

The long-term fasting experiment, carried out in the laboratory, used 20 males and 20 females from each of the 3 sampling sites. The breeding conditions were the same as those described for the short-term fasting experiment with the exception that the snails were kept individually and not in couples. The snails were kept in their aquariums without any food and the water was changed once a week. The initial shell length and live weight were used as above to estimate the initial condition factor (CF, g/mm). The live weight and the survival of the snails, and the number of eggs and egg masses in the case of the females, were recorded weekly. The experiment finished after 33 weeks with only one female specimen from population C

remaining alive. The survival time (ST, days until death) was studied using Kaplan–Meier method and Tarone–Ware tests. The relative change in weight between the first and the last week before death were analyzed. Condition factor and relative change in weight was studied using Kruskal–Wallis to test differences among the 3 populations and Mann–Whitney *U* test was used for comparisons between populations or sexes.

The effects of long-term fasting in hatchlings

The objective of the hatchlings long-term fasting experiment was to study the possible interpopulational variation in the survival of the hatchlings under long-term absolute fasting. Twenty egg masses were collected in the field from each of the 3 aforementioned populations. These clutches were incubated at $25^{\circ}C$ in the laboratory until hatching. Ten hatchlings from each egg mass were isolated within 24 hours of hatching in a plastic jar with 200 cm^3 of tap water at $25^{\circ}C$. The water in these jars was renewed every week; the hatchlings were kept without any food; and their survival was recorded every week (Tamburi and Martín 2011). The differences between the 3 populations in the maximum survival time of each egg mass were analyzed with one-way ANOVA for each aquarium.

Results

The effects of short-term fasting in adults

The survival distribution showed differences in male snails exposed to different extents of fasting (Figure 1A; Tarone–Ware test = 11.97; $P < 0.01$; $df = 3$), whereas no differences were found in the survival distribution of females (Figure 1B; Tarone–Ware test = 3.91; $P > 0.25$; $df = 3$). The overall mortality was higher in the males than in the females (Tarone–Ware test = 5.35; $P < 0.05$; $df = 1$).

The copulation activity of the snail couples decreased under fasting, and it began to recover when the food supply was restored (Figure 2A). An overall copulatory activity of 57% was observed during the feeding period, whereas this value was 19% during fasting for all treatments. These differences were highly significant ($\chi^2_3 = 15.18$, $P < 0.01$). The main difference was between the control and the treatments as no significant differences were seen between the treatments in the last week of fasting ($\chi^2_2 = 3.88$, $P = 0.14$).

The number of females laying eggs during the week (Figure 2B) dropped abruptly under fasting and began to recover rapidly when the food supply was restored. An average of 70% of the females laid

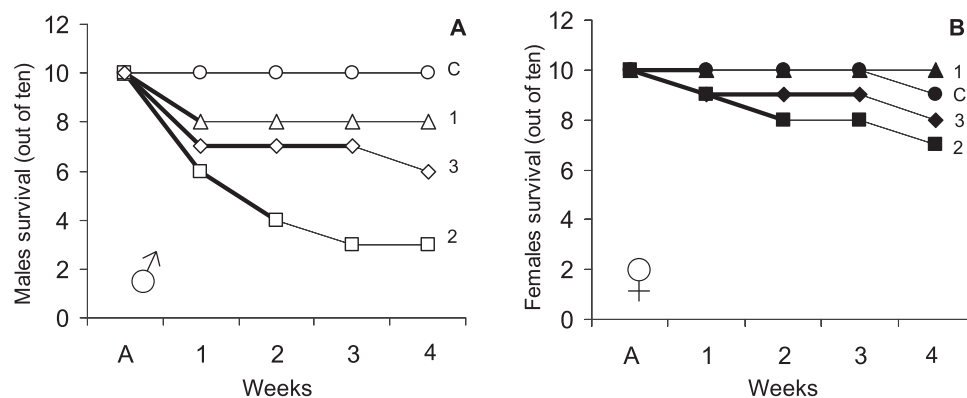


Figure 1. The effect of short-term fasting on the survival of *P. canaliculata* adult snails: (A) males, (B) females. The thick lines represent the fasting period and the thin ones periods with food *ad libitum* (A: acclimatization week). Treatments: C: control, 1: 1 week of fasting, 2: 2 weeks of fasting, and 3: 3 weeks of fasting.

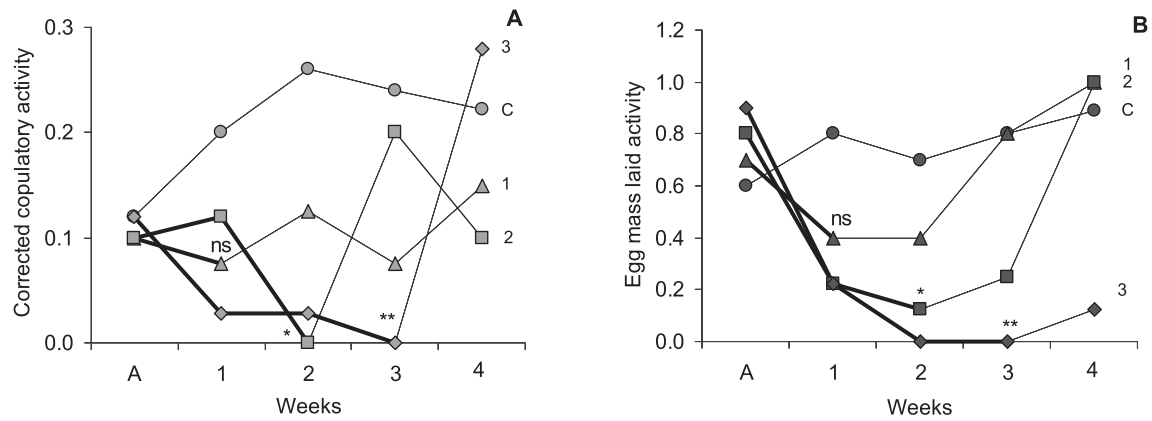


Figure 2. Effect of short-term fasting periods on the reproductive activity of *P. canaliculata*. (A) corrected copulation activity, (B) egg-laying activity. The thick lines represent the fasting period and thin ones periods with food *ad libitum* (A: acclimatization week). Treatments: C: control. 1: 1 week of fasting, 2: 2 weeks of fasting, 3: 3 weeks of fasting. ns, *, and ** indicate the result of testing the treatment of 1, 2, or 3 weeks of fasting against the corresponding week of the control.

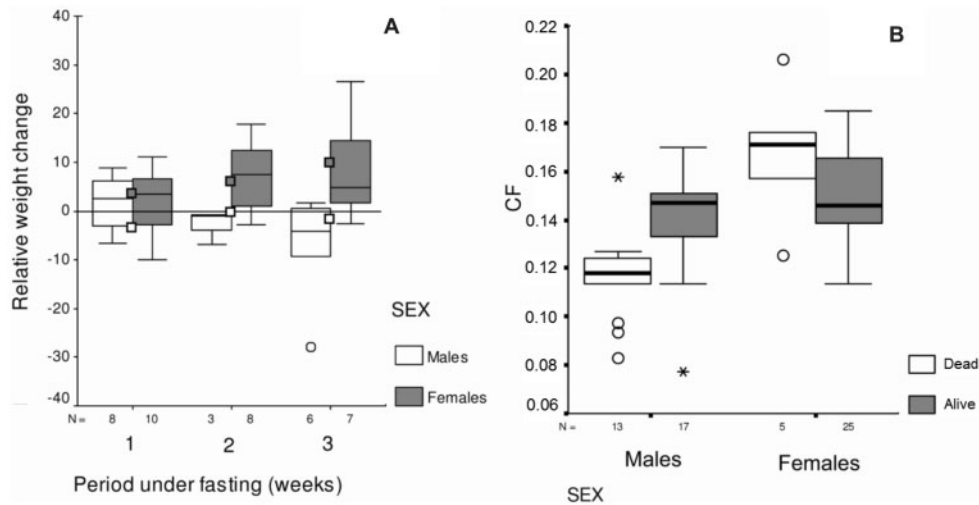


Figure 3. Growth and somatic state of *P. canaliculata* during short-term fasting experiment (boxplots: circles and stars represent data that deviate more than 1.5 and 3 interquartile ranges from quartile 1 or 3.) (A) Relative change in weight during fasting. The small squares represent the mean value for relative change in weight for the control (continual food supply), calculated for the same period of time as the treatments (1, 2, and 3 weeks of fasting). (B) Initial condition factor (CF = $\log_{10} (LW_i / SL_i) + 1$) of the snails used in the experiment according to their survival in the 4-week experiment; the graph included all the treatments with some degree of fasting (i.e., 1, 2, and 3 weeks of fasting).

eggs during the feeding period regardless of the treatment, whereas only 16% did so under fasting. These differences were highly significant ($\chi^2_3 = 10.34$, $P = 0.016$). An average of 226 ± 156.7 (mean \pm SD) eggs per female per week was observed in the snails with food in their aquariums, regardless of the treatment, but only 14 ± 23 eggs per female were deposited under fasting.

Females showed an increase in relative weight change (Figure 3A; $t_{24} = 3.486$; $P < 0.01$) with increased fasting, while males exhibited a decrease or lack of a change ($t_{16} = -1.104$; $P = 0.286$), resulting in a significant statistical interaction ($F_{2,36} = 3.636$; $P = 0.036$) between sex and duration of fasting.

The mortality of the females with at least some degree of fasting during the experiment did not show any relationship to the initial condition factors (Figure 3B; t -test $t_{28} = 1.733$, $P = 0.094$). On the other hand, the initial condition factor of the males that died was lower than of those that survived (Figure 3B; one-tailed t -test $t_{28} = -3.002$, $P < 0.01$).

The effects of long-term fasting in adults

The relative abundances estimated in the field, calculated as the number of snails per unit of effort, were similar for the populations A and C and higher than in population B (Table 1). The sexual dimorphism in size at maturity (first quartile of shell length) was minimum in population B, while it was quite higher and similar in A and C populations.

In the laboratory experiment, differences in the survival time were found among the populations (Figure 4, Tarone-Ware test = 25.4; $P < 0.01$; $df = 2$). There were significant differences between population A and populations B and C (Mann-Whitney U test, $z = -2.817$, $z = -4.548$, respectively; Dunn-Sidak global error for both comparisons, $P < 0.01$), whereas no significant differences were found between populations B and C (Mann-Whitney U test, $Z = 1.747$, $P = 0.081$). We found no significant differences between sexes for any of the 3 populations (Tarone-Ware statistics ranging from 0.00 to 2.01, with P values of 0.98 and 0.16, respectively). Only 12 events of oviposition by 8 females were observed in

Table 1. Features of the 3 populations studied (A, B, and C) in the long-term fasting experiment: first quartile of shell length (mm) of copulating females and males and relative abundance (snails/minute) in each population

| Population | Females first quartile | Males first quartile | Relative abundance |
|------------|------------------------|----------------------|--------------------|
| A | 36.9 | 32.1 | 4.67 |
| B | 42.7 | 42.2 | 1.77 |
| C | 36.8 | 32.8 | 4.54 |

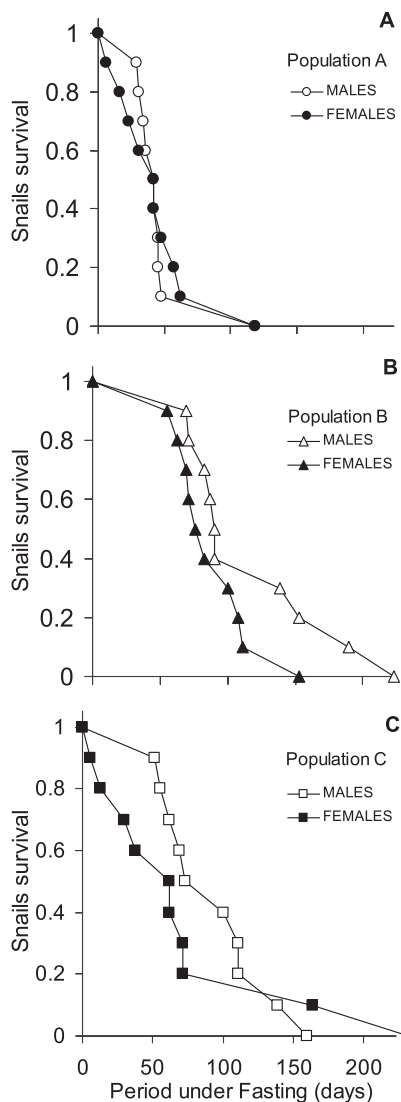


Figure 4. Survival curves of the snails in the long-term fasting experiment, according to source population and sex. (A) population A; (B) population B; (C) population C.

treatments A and B. The last egg mass was recorded 20 days after the beginning of the experiment.

The relative change in weight during the experiment was not significantly different between the populations (Kruskal–Wallis $\chi^2_2 = 1.05$, $P = 0.581$). The percentage decrease in the males was significant (-4.23% ; $t_{27} = -2.92$, $P < 0.01$), but it was not significant in the females (-3.32% ; $t_{28} = -1.7$, $P = 0.095$). Differences were

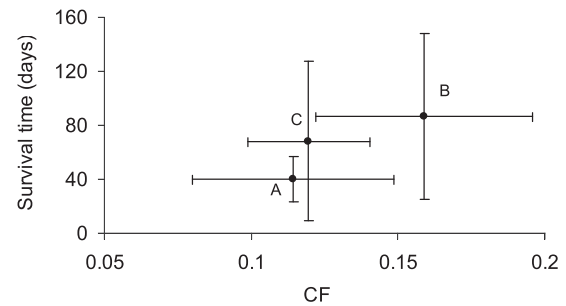


Figure 5. Condition factor ($CF = \log_{10}(LW_1/SL_1) + 1$) and survival time in each of the 3 populations sampled (A, B, and C). The central values are the means, and the bars the interquartile range.

found in the condition factor among the populations (Figure 5, Kruskal–Wallis $\chi^2_2 = 21.15$, $P < 0.01$). Population B showed significant differences with A and C (Mann–Whitney U test, $z = -3.92$, $z = -3.95$, respectively; Dunn–Sidak global error for both comparisons, $P < 0.01$), whereas no significant differences were found between the populations A and C (Mann–Whitney U test $z = -0.866$, $P = 0.398$). No differences were detected between the sexes in the condition factor in population B (Mann–Whitney U test $z = -0.529$, $P = 0.597$) nor in C (Mann–Whitney U test $z = -1.587$, $P = 0.112$), but differences were found in A (Mann–Whitney U test $z = -2.192$, $P = 0.028$).

The effects of long-term fasting in hatchlings

No differences were found in the maximum survival time of the hatchlings in Experiment 3 (one-way ANOVA $F_{2,57} = 2.073$, $P = 0.135$). The mean maximum survival time of all hatchlings from each egg mass was 52.6 days.

Discussion

Our results demonstrate that abrupt fasting in the short term leads to rapid restriction of reproductive activity (copulation and oviposition) in *P. canaliculata*. Short-term fasting produces a notably higher mortality in the males. A lower initial condition factor was found in the males that died during the experiment, whereas this was not observed in the females. The males of *P. canaliculata* have a smaller digestive gland than the females (Vega et al. 2006), and their specific ingestion rates and food conversion efficiencies are lower (Tamburi and Martín 2009b), so it is likely that their energy reserves reach a critical level more frequently.

In the long-term fasting experiment, of the 3 populations analyzed, population B was the one that showed most signs of greater food availability: the relative abundance of this population was lower and the copulating animals were larger than in populations A and C. On the other hand, the difference in the minimum sizes of mating males and females is very small, which coincides with the prediction that there is less sexual size dimorphism when the food availability is greater (Tamburi and Martín 2009a). Population B was also the one with the highest macrophyte coverage in previous studies within this basin (Seuffert and Martín 2013). These different degrees of environmental food availability result in variation in the condition factors (Tamburi and Martín 2011) and that in turn is likely to result in different survival times when fasting.

The higher mortality of the males when maintained fasting with females (short-term fasting experiment) appears to be related to a smaller quantity of reserves and lower condition factor. In the long-

term fasting experiment, in which the snails were completely isolated, a higher mortality was not seen in the males. One possible explanation is that the males maintain mate-searching or copulatory behavior even under conditions of tropic stress, which results in the loss of much energy and so critical levels are exceeded in the males with lower reserves. The males of *P. canaliculata* are more reckless and are less likely to restrain their activity in the presence of predators than the females (Xu et al. 2014). This suggests that males prioritize the search for mates over survival under conditions of tropic stress or high predation risk. This is consistent with a scramble strategy in the competition of males over mates (Andersson and Iwasa 1996) that mature as soon as possible independently of the food availability (Tamburi and Martín 2009a) and that show a trade-off between somatic growth and sperm production (Tamburi and Martín 2009b).

There were no significant changes in the weight of males during fasting (short-term fasting experiment), but there was a notable increase in the weight of fasting females. Calcium carbonate deposition in the shell continues for a time in *Pomacea glauca* (Linnaeus 1756) even during fasting (Zischke et al. 1970). On the other hand, the weight increase may be due to the consumption of low density storage substances (lipids) and its replacement for water in the tissues. However, none of these 2 effects may explain the differences between sexes. The albumen gland, which has a high content of calcium carbonate for the formation of egg shells (Catalán et al. 2002), may continue concentrating it under the calcium carbonate-saturated water conditions in our study. This would explain the increase in weight during the fasting period of up to 3 weeks only for the females. In the long-term fasting experiment, the weight of the snails decreased 3.77% before they die, indicating that after 3 weeks of fasting the loss of biomass overrides the weight increase by calcium carbonate deposition.

In the fasting treatments, the variables related to reproduction (copulation and oviposition) fell abruptly at the beginning of fasting, which is different from that observed when the snails are bred with a constant food deficit (Tamburi and Martín 2011). The females that are bred since hatching with chronic deficit of food availability of up to 80% lay fewer eggs than the females bred *ad libitum*, but they do not discontinue their reproductive activity (Tamburi and Martín 2011) which has also been observed in adult females under low constant food availability (Albrecht et al. 1999; Tanaka et al. 1999). The cessation of reproductive activity seen here is reversible if the food supply is restored within a few weeks. This strategy of the females prioritizes their survival and may allow them to survive interruptions or very large decreases in food availability and then to reassume reproductive activity.

A high survival in *P. canaliculata* hatchlings whose mothers were bred with some degree of food deprivation was observed in a previous study (Tamburi and Martín 2011). However, no differences were detected in the survival time of hatchlings from these 3 populations with different food availabilities studied here. Perhaps the variation in the food availability in the natural populations was not as great as those created in the laboratory (100% to 20%), which could add to other uncontrolled factors affecting the mothers and the embryos before collecting the egg masses to mask the maternal effects.

In spite of the aforementioned differences in the 3 populations studied, between 10% and 40% of the starving adult snails survived for more than 100 days and 3.3% lasted for over 200 days. Even the starving hatchlings were capable of surviving fasting for an average of 52.6 days and up to a maximum of 81 days. Although these

values are less than those reported for *P. canaliculata* snails out of water (Fan et al. 2000; Yusa et al. 2006), they are however significant in the ability of *P. canaliculata* populations to persist and in the possibilities of dispersal. Our results indicate that great numbers of hatchlings could survive unnoticed for 2 months in freshwater ballast tanks. Adult snails can also subsist for months without food in pipelines, artificial channels, or water tanks. One single fertilized female that survives after being transported could lay thousands of viable eggs due to their ability of storing sperm (Estebenet and Martín 2002; Burela and Martín 2011) and could initiate a new population (Jerde et al. 2009). The ability of this invasive apple snail to resist fasting should be taken into account when designing quarantine schedules to control its spread and establishment.

Funding

This study was funded by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET; PIP 112-200901-00473), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT2012-1956) and Universidad Nacional del Sur (PGI 24/B185). NET and PRM are researchers in CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas).

Acknowledgments

We are grateful to Silvana Burela for sharing information relative to the populations of snails on December 2013 and to Enzo Manara, Fernanda Gurovich and Lucía Saveanu for their help with the fieldwork. We wish to thank to Dr. Yoichi Yusa and an anonymous reviewer for their helpful and constructive comments.

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