Survival, recovery, and reproduction of apple snails (Pomacea spp.) following exposure to drought conditions

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Abstract: Climate change has dramatically altered freshwater ecosystems and will continue to affect them further. As water-level fluctuations increase in frequency and intensity, the tolerance of aquatic organisms to abiotic stressors will become critical determinants of survival. Apple snail species in the genus Pomacea (Ampullariidae) live in freshwater throughout the tropics and subtropics, but deposit egg clutches on terrestrial substrates. Two species, Pomacea canaliculata and Pomacea maculata, are amongst the world’s most invasive species and are expanding their invasive ranges. To evaluate their survival during extreme dry-down conditions in their native range, we collected 156 apple snails (Pomacea spp.) from 4 permanent water bodies in Uruguay and buried them in dry sand to simulate drought. We placed the snails outside, sheltered from sun and rain (ambient temperature: 15.2–28.1°C, ambient humidity: 44.9–100.6% below sand surface, 34.2–98.7% in outside air), and checked survival daily. Genetic identification confirmed the presence of P. canaliculata, P. maculata, and a morphologically indistinguishable undescribed congeneric among these snails. Thirty-six percent of snails survived the entire period (47 d) buried in the sand. Fewer males survived than females. Median lethal exposure time (LT50) was 33 d for the total group of Pomacea species. After re-immersion in water, 93% of survivors resumed normal activities, including feeding, within 24 h and mated during later observations, indicating resistance to drying conditions and resilience during recovery. Offspring from 1 clutch laid after recovery survived >1 y after this experiment. We found high tolerance to prolonged drying and rapid recovery after re-immersion for 3 Pomacea species. As drought frequency increases with climate change, tolerance to these conditions and rapid restoration of normally high reproductive rates after dry-down may confer an advantage to these species, contribute to long-term persistence in their native range, and facilitate continued invasions.

Key words: Ampullariidae, climate change, water-level fluctuations, resilience, invasiveness

Over the coming decades, climate change probably will match or surpass habitat destruction as the greatest contributor to biodiversity loss (Bellard et al. 2012). Extended periods of drought will become more frequent and severe in many regions of the world (Bogan et al. 2015). Drought will accelerate species losses, alter species ranges, and cause physiological stress for many others, thereby reducing biodiversity in freshwater systems (Wrona et al. 2006, Kundzewicz et al. 2008, Jeppesen et al. 2014). Climate change is expected to affect freshwater macroinvertebrates disproportionately (Cordellier et al. 2012) through severe alterations in temperature regimes and declines in water quality and water level (Galbraith et al. 2015, Mouthon and Daufresne 2015, Lund et al. 2016).

Effects of water-level fluctuations remain understudied in most permanent aquatic ecosystems (Coops et al. 2003, Wantzen et al. 2008, Jeppesen et al. 2015, Evtimova and Donohue 2016) and across many invertebrate taxa (Gal-
the adaptations necessary to tolerate abiotic stressors that might otherwise prevent invasion (Kolar and Lodge 2001). However, investigators who have characterized tolerance to drying conditions for select species of Pomacea (Darby et al. 2008, Yoshida et al. 2014, Watanabe et al. 2015) have relied primarily on aerial exposure, and only Darby et al. (2008) provided substrate that allowed the snails to burrow during drying conditions. We fully buried the snails in our study and analyzed their response to drying conditions in a simulated dry-down experiment within their native range. We tested the ability of surviving snails to resume normal behaviors and reproduce after simulated drought followed by re-immersion. Our goal was to assess whether they simply survive drought or fully recover. A more comprehensive understanding of how these snails tolerate and recover from environmental stressors may help explain their evolutionary and invasive success (Hayes et al. 2009a) and may help scientists anticipate molluscan responses to climate change.

**METHODS**

**Exposure to drying conditions**

We collected apple snails during the austral summer (17–22 December 2014) from 4 populations in Uruguay (Table 1), including 2 shallow lakes near Maldonado (Laguna del Diario and Laguna del Sauce) and 2 small lakes in city parks, Parque Rodó of Montevideo and Parque Rodó of San José de Mayo. We weighed each snail and measured operculum width and shell height (Youens and Burks 2008). We collected snails of diverse sizes (1.2–43.2 g fresh mass) from all sites. Shell heights ranged from 10.6 to 43.0 mm. Operculum width, a more reliable measure (Youens and Burks 2008), ranged from 13.3 to 48.2 mm. Removal from water and drying induces aestivation in Pomacea species (S. Thiengo, FIOCRUZ, personal communication), so we wrapped snails in paper towels, which also absorbed liquid from wastes, and stored them in plastic bags for 40 h prior to the experiment. This pre-experimental acclimation also enabled us to remove snails near mortality before the experiment, and reduced the likelihood of including any snails inadvertently damaged during collection and transport.

We measured each snail and identified its sex (when possible) based on operculum morphology and by checking through the shell for a visible albumin gland (to identify mature females) or testes (to identify mature males). In cases of small snails (typically operculum width < 20 mm), we were unable to identify the sex prior to the experiment. In those cases, we identified sex at the time of death by anatomical examination. In some cases, anatomical examination revealed that the snail had not matured sufficiently to possess sexual organs. We designated these snails as juveniles in subsequent analyses.

We collected sand from a coastal location in Maldonado, Uruguay. We removed organisms and debris and
dried the sand outside for ≥24 h before using it in the burial experiment. We lined 6 plastic grate boxes (40 × 60 × 16 cm) with plastic to prevent moisture and invertebrates from entering the bottom and filled the boxes with sand to ~2 cm from the top. We buried 20 to 30 individually labeled snails in each box such that each snail had a layer of sand ~0.5 cm thick between its body and open air and marked the location of each snail with a small flag. We placed fine plastic mesh over the top of each box to permit air flow, prevent insects from entering, and deter predation by birds or mammals.

We conducted the experiment outside to permit exposure to natural atmospheric conditions (i.e., temperature, humidity), and sheltered the boxes with a tarp to prevent rain from wetting the sand and prematurely ending aestivation. We checked temperature and relative humidity beneath the sand and in the outside air with a thermohygrometer (HI 93640; Hanna Instruments, Woonsocket, Rhode Island). Temperatures ranged from 15.0 to 29.3°C in the sand and from 15.2 to 28.1°C in the air. Relative humidity ranged from 44.9 to 100.6% in the sand and from 34.2 to 98.7% in the air. On each day of the experiment, we checked each snail for signs of death (e.g., fluid accumulation near the snail, a distinctive odor of death [sniff test], or lack of resistance when the operculum was pulled). We removed all dead snails and recorded the date of death.

**Recovery**

We ended the outdoor exposure after 47 d, when ≥50% of the experimental population had died. On day 48, we removed survivors, washed them, and placed them in individual aquaria (20 × 9.5 × 15.5 cm) with ~2.5 L of water. We recorded several behaviors (following Giraud-Billoud et al. 2011) 5, 15, 30, 60, and 90 min post-immersion. Behaviors were: aperture sealed with the operculum, aperture agape, first tentacle extension, foot deployment, attachment to or crawling on the aquarium floor and walls, and feeding. After 90 min, we added fresh lettuce to each aquarium and recorded behaviors 2, 3, 5, 9, 18, 20, and 24 h post-immersion. After 24 h of immersion, we grouped snails by collection site and moved them to 4 larger aquaria (35 × 25 × 50 cm) with ~30 L of water. We continued observations every 8 h for 4 d to record mating. We monitored for oviposition daily for 3 wk after immersion and intermittently thereafter.

**Species identification**

We initially identified *P. canaliculata* as the only species present in our populations based on museum records, past research, and shell and penis sheath morphologies (Hayes et al. 2008, 2012). However, substantial diversity exists in the genus *Pomacea* (Hayes et al. 2009b), and species have often been misidentified (Hayes et al. 2012). Therefore, we sampled foot tissue from 36 recently dead snails for genetic identification. We collected tissue samples during the first 20 d of the desiccation experiment and stored them in 95% ethanol.

We used the NucleoSpin® tissue extraction kit (Macherey–Nagel, Bethlehem, Pennsylvania) to extract genomic DNA from ~5 mg of preserved foot tissue. We amplified a 709 base pair (bp) fragment of the mitochondrial cytochrome c oxidase subunit I locus (COI) following Hayes et al. (2009b). We checked amplified products for quality and specificity via gel electrophoresis and sent amplicons for sequencing at the Institute for Cellular and Molecular Biology at the University of Texas at Austin, USA. Cycle sequencing was done with ABI BigDye terminator kits (Perkin–Elmer Applied Biosystems, Foster City, California) and sequences were electrophoresed and analyzed on an ABI 3730XL (Perkin–Elmer Applied Biosystems). Electropherograms were checked by eye for errors and ambiguous calls, and contiguous sequences were assembled in Geneious (version 7.1; Kears et al. 2012).

We used National Center for Biotechnology Institute (NCBI) Basic Local Alignment Search Tool (BLAST) (Johnson et al. 2008) and comparison with sequences from other conspecifics and congenerics (Hayes 2009) to verify that the correct locus was sequenced. All unique sequences and those of other *Pomacea* spp. from Hayes et al. (2009b) were aligned using MUSCLE (version 3.6; Edgar 2004) as a plugin in Geneious. The most appropriate nucleotide substitution model was selected using jModelTest (version 2.1.7; Darriba et al. 2012) and Akaike’s Information Criterion (AIC). We estimated phylogenetic relationships of our samples based

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### Table 1. Composition of experimental snail communities. Juveniles are snails that had not reached sexual maturation. Genetic samples were primarily *Pomacea canaliculata* and *Pomacea maculata*, with the cryptic congeneric (*Pomacea sp.*) also identified at Parque Rodó San José.

<table>
<thead>
<tr>
<th>Site</th>
<th>Source</th>
<th>Species confirmed</th>
<th>Latitude/longitude</th>
<th>Total</th>
<th>Male</th>
<th>Female</th>
<th>Juvenile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laguna del Diario</td>
<td>Lake</td>
<td><em>P. canaliculata</em></td>
<td>34°54′31.6″S/55°0′46.6″W</td>
<td>28</td>
<td>9</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Laguna del Sauce</td>
<td>Lake</td>
<td><em>P. canaliculata</em></td>
<td>34°50′14.4″S/55°9′24.0″W</td>
<td>24</td>
<td>10</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Parque Rodó San José</td>
<td>Pond</td>
<td><em>P. canaliculata, P. maculata, Pomacea sp.</em></td>
<td>34°19′8.68″S/56°43′7.02″W</td>
<td>56</td>
<td>8</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Parque Rodó Montevideo</td>
<td>Pond</td>
<td><em>P. canaliculata, P. maculata</em></td>
<td>34°54′31.1″S/56°10′22.0″W</td>
<td>48</td>
<td>29</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>156</td>
<td>56</td>
<td>76</td>
<td>24</td>
</tr>
</tbody>
</table>
on maximum likelihood with the HKY+I+G model in PhyML (version 3.0; Guindon et al. 2010) and estimated node support with 500 bootstrap replicates (Felsenstein 1985). We assigned species identities by the grouping of our samples in well-supported, monophyletic clades that contained sequences from previously identified species (Hayes 2009, Hayes et al. 2009b).

Statistical analysis
To test how sex and reproductive development influenced survival, we grouped snails into categories based on our assessment of their reproductive development. We classified reproductively mature individuals as male or female, and we considered a combined category, adults. Snails that were not sexually mature were categorized as juveniles. We used Kruskal–Wallis (KW) tests followed by Dunn’s tests for pairwise comparisons to evaluate differences in size (operculum width) or mass between reproductive development categories. We used 2 dependent variables for analyzing resistance to drying conditions: number of survival days for each individual and proportion of survival within category over time.

To examine survival within groups, we considered rate of death by tracking the total number of deaths within each category of reproductive development on each day and used this number to calculate % daily survival. We used analysis of covariance (ANCOVA) to test for effects of size on survival related to category of reproductive development (male, female, juveniles) by specifying operculum width and mass as covariates for the dependent variable (days of survival). For these 2 ANCOVAs, we assigned snails that survived the full 47-d exposure a survival time of 48 d.

To evaluate mortality rates across categories, we used linear regressions to test relationships between % daily survival and time. From these regressions, we extrapolated the time at which 50% of a group was expected to die from exposure to drying conditions (LT50). We then followed the procedures published by Wheeler et al. (2006) to generate 95% confidence intervals (CIs) for each LT50. We compared CIs between categories of reproductive development to assess whether they overlapped. We also used ratio tests as a more sensitive comparison of LT50 values (Wheeler et al. 2006). In this approach, the ratio of 2 log-transformed LT50 values (LR, or log ratio) is used to calculate a z-score equal to (LR)/(SE|LR|), where SE is the standard error of LR. We calculated the ratio test and CIs in Microsoft Excel® (version 14.4.6; Microsoft, Redmond, Washington) and performed all other statistical analyses with SPSS (version 22.0; IBM, Armonk, New York).

RESULTS
Exposure to drying conditions
Apple snails in our experiment showed considerable ability to survive the drying conditions created by full burial. Survival was 35.9% after 47 d (Table 2). Survival varied among categories of reproductive development (Fig. 1). More females (44.7%) survived than males (32.1%). This effect did not appear to be related to size or mass. Operculum width and mass did not differ between male and female adults (Dunn’s test, width: Z = –9.14, p = 0.251; mass: Z = –6.1, p = 0.443). Days of survival did not vary among categories of reproductive development (ANCOVA, \( F_{1,152} = 0.510, p = 0.602 \)) when considering mass as a covariate \( (F_{1,152} = 0.268, p = 0.887) \). Days of survival also did not vary among categories of reproductive development (ANCOVA, \( F_{1,152} = 0.268, p = 0.605 \)) when considering operculum width as a covariate \( (ANCOVA, F_{2,152} = 0.497, p = 0.609) \).

Juvenile % survival declined more rapidly than % survival of sexually developed adult snails in the last days of the experiment (Fig. 1). Operculum width, shell height, and mass varied significantly between juveniles and the total group \( (KW, X^2_{2,156} = 49.63, 48.45, 49.76, \text{ respectively),} \)
all \( p < 0.001 \). Juveniles weighed significantly less than adult males and females (\( \sim \frac{1}{3} \) of adult mass; Dunn’s test, \( Z = -73.81, -67.7, \) both \( p < 0.001 \)), but a broad range in sizes resulted in overlapping size distributions across reproductive categories.

The LT\(_{50}\) for our total group of 156 snails was 33.2 d (Table 2). Females had the highest expected survival (LT\(_{50}\) = 37.4 d). The CIs of LT\(_{50}\) values for females did not overlap with the CIs of LT\(_{50}\) values for males or juveniles (Table 2). However, based on the ratio test, LT\(_{50}\) values did not differ between females and males (\( Z = 1.55, p = 0.124 \)) or between females and juveniles (\( Z = 1.57, p = 0.120 \)). In all other comparisons between categories of reproductive development, CIs for LT\(_{50}\) values overlapped and ratio tests were not significant (Table 2).

**Recovery**

Fifty-six (36%) of 156 snails survived the 47-d experiment. Of these, 52 snails (93%) survived re-immersion and resumed normal behavioral patterns (Fig. 2). Recovery was not immediate and the transition out of aestivation progressed gradually. Most of the surviving snails resumed normal behaviors in a progressive sequence, from unsealing the aperture and resting agape to extending a tentacle and beginning movement (Fig. 2). As the recovery phase progressed, more snails became active (e.g., crawling). Most snails (81%) fed within 24 h of re-immersion. At 5 min, 87% of the snails remained sealed. After 15 min, 44% of snails were agape, but 56% remained sealed. Only 23% of snails remained sealed after 30 min. By 90 min, 98% of snails were agape. After 3 h, all living snails showed signs of activity and the first observed instances of feeding (4%) occurred, 90 min after lettuce was made available (Fig. 2).

We observed 4 instances of mating during 4 d of subsequent observation. Mating pairs were observed among snails from Laguna del Diario, Laguna del Sauce, and Parque Rodó Montevideo. We did not observe mating among the survivors from Parque Rodó San José, the population with the lowest rate of survivorship and numerous juvenile snails. Between 33 and 42 d after re-immersion, 3 snails (2 from Laguna del Sauce and 1 from Parque Rodó Montevideo) deposited eggs on the side of aquarium above the water line. The clutch from Parque Rodó Montevideo hatched after 23 d. Some of these juveniles survived and grew in the laboratory for 1 y before we preserved them as vouchers. The other 2 clutches failed to hatch, probably because the embryos died when they fell into the water after becoming detached from the side of the aquarium (Pizani et al. 2005).

**Species identification**

Phylogenetic analyses of the individuals sequenced recovered all 36 samples in 3 well-supported monophyletic clades (not shown) matching 3 species: *P. canaliculata* (\( n = 22 \)), *P. maculata* (\( n = 4 \)), and *Pomacea sp.* (\( n = 10 \)), the latter being an undescribed species of *Pomacea* previously recorded in Brazil and Uruguay (Hayes 2009). We found *P. canaliculata* at all 4 sites, but disproportionately represented (100%) in our tissue samples from the lakes Sauce...
DISCUSSION

Our results provide experimental evidence that Pomacea spp. were able to survive extended periods of drying in conditions that simulated their natural burrowing behavior and allowed them to aestivate (Hayes et al. 2015). Females survived longer than males, a finding that is important in terms of population persistence. The vast majority of surviving adults (93%) also survived re-immersion and resumed normal activities within 24 h of their return to water. Giraud-Billoud et al. (2011) recorded similar behavioral recovery of P. canaliculata individuals. We further observed that snails of the Pomacea species in our study resume mating and can reproduce successfully after extended exposure to drying conditions. Invasive Pomacea species produce large numbers of eggs with high hatching efficiency (Barnes et al. 2008, Kyle et al. 2013). Thus, the presence of mating females among the survivors all but guarantees replacement of a population as long as a few offspring survive. Even if only a small number of females survive, the population will continue to grow and may reach a nuisance stage in nonnative habitats (Keller et al. 2007).

Supraseasonal droughts of longer duration than typical seasonal droughts may extinguate many aquatic species (Bogan et al. 2015). Our study points toward high tolerance of several Pomacea species to drying conditions that frequently occur in their native and nonnative habitats and that are likely to occur more frequently in the future (Bellard et al. 2012, Jeppesen et al. 2014, Lund et al. 2016). Tolerance to abiotic stressors probably contributes to the long-term success of these Pomacea species in their native ranges and provides some of the essential traits of a successful invader (Kolar and Lodge 2001). In the event of such droughts in their nonnative habitats, surviving Pomacea species may gain a competitive advantage over native species. The general tolerance of 3 distinct species to dessication documented by our study suggests an evolutionary history (Hayes et al. 2009a) that probably has contributed to the evolutionary and invasive success of apple snails (Lowe et al. 2000, Hayes et al. 2009a, 2012).

Unfortunately, our limited genetic identification leaves open the question of species-specific differences that warrant future investigation. Furthermore, the unexpected presence of a cryptic species among our samples underscores the key issue of cryptic diversity and the importance of using an integrative systematics approach in freshwater ecology (Pauls et al. 2014). However, survival of apple snails (LT50 and % survival) in our study exceeded that of aerially exposed Pomacea spp. (Yoshida et al. 2014) for all categories of reproductive development except juveniles. Yoshida et al. (2014) found 0% survivorship for 2 P. maculata cohorts and 1 P. canaliculata cohort, and 18% survivorship among 1 group of P. canaliculata at 48 d. These values are substantially lower than the 36% survival at 47 d in our study. In contrast, Watanabe et al. (2015) found an average survival time for aerially exposed P. maculata (given as P. amazónica, a synonym of P. maculata; Hayes et al. 2012) of 42.9 ± 24.7 d (SD). They also found survival rates of ~40% at 50 d, values similar to our overall survivorship of 36% after 47 d. Differences in exposure method, snail size, and species can contribute to differences in results among studies.

The high survival rate that we observed among apple snails during dry conditions is best explained by their diverse suite of behavioral and physiological adaptations for tolerating abiotic stressors. For example, Pomacea paludosa (Say, 1829), native to Florida, had a 70% survival rate after 12 wk (84 d) in a comparable simulated outdoor drydown event (Darby et al. 2008) during which most snails burrowed. In another study, P. paludosa moved toward deeper water when the water was 10 to 20 cm deep, but moved less when the water was <10 cm deep (Darby et al. 2002). This type of migration, when paired with burrowing behavior, can facilitate prolonged resistance to drying conditions for snails (Poznańska et al. 2015a). Burrowing also can reduce the threat of predation (Stevens et al. 2002). As counter-measures to limited mobility, diverse traits such as a flexible operculum, burrowing behavior, and laying eggs above water all probably contribute to the success of several Pomacea species, both in their native and invaded ranges (Hayes et al. 2009a, 2015). Combined physiological adaptations and behavioral responses contribute to drying tolerance of mollusks as a whole (Gough et al. 2012, Poznańska et al. 2015b) and underscore the amphibious nature of apple snails (Hayes et al. 2009a).

These adaptive traits and behaviors accompany altered metabolic processes that facilitate survival during aestivation. Giraud-Billoud et al. (2011) demonstrated that increased antioxidant production accompanied aestivation in apple snails, and Sun et al. (2013) showed that P. canaliculata altered gene expression during aestivation to increase lipid metabolism, slow protein degradation, and prevent oxidative tissue damage. More generally, reducing ATP metabolism by deactivating energy-intensive ion transport also helps snails sustain the hypometabolic state of aestivation (Ramnanan and Storey 2006, 2008). A better understanding of the physiological mechanisms that govern aestivation may enable investigators to model the environmental conditions necessary to sustain specific species bioenergetically (Sokolova et al. 2012), thereby improving predictions of species ranges during climate change and invasion.

Diverse mechanisms for withstanding and responding to environmental stressors will play a large role in determining diversity and distribution of species in the face of continued climate change (Woodward et al. 2010, Bellard et al. 2012, Jeppesen et al. 2014). Our results reflect the
same rapid post-drought recovery that Bogan et al. (2015) found in a review of macroinvertebrate community responses in arid-land streams. Population fluxes in herbivorous species dependent on macrophytes, such as apple snails, may lead to shifts in alternative states (Carlsson et al. 2004) and, consequently, influence links between pelagic and benthic production (Jeppesen et al. 2014) in lake systems. Most investigators who examine tolerance to drying focus on intermittent or semipermanent riverine systems, but water-level fluctuations also strongly affect shallow lakes and ponds (Jeppesen et al. 2015). Evtimova and Donohue (2016) found altered taxonomic and trophic structure of benthic consumers in lakes that undergo high water-level fluctuations. Ecological disruptions, such as droughts, can lead to changes in community structure (Mouthon and Dufrénot 2015) and increase the likelihood of extirpation for low-motility or nonmotile taxa, such as flightless insects (Bogan et al. 2015) or bivalves (Kappes and Haase 2012). In contrast to other low-mobility taxa, *Pomacea* species tolerate such disruptions, and their ability to recover and reproduce may confer an advantage over other species.

Tolerance to environmental stressors overall has been implicated in the success of NNIS, especially of freshwater mollusks (Havel 2011, Weir and Salice 2011). Water temperature and pH predict the possible range of *P. maculata* invasion in the USA (Byers et al. 2013), whereas tolerance to cold temperatures during winter aestivation seems to determine the invasive ranges of each species in Japan (Matsukura et al. 2016). As drought frequency increases with continued climate change in many regions (Woodward et al. 2010, Cordellier et al. 2012), the high tolerance to and ability to recover from drying conditions may confer advantages to apple snails, relative to alternate life-history strategies used by other freshwater invertebrates (Strachan et al. 2015). Thus, tolerance and resistance to drying and high population resilience after may partially account for the success of *Pomacea* spp. as expanding global invaders (Kolar and Lodge 2001, Horgan et al. 2014).

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**LITERATURE CITED**


