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# THE EFFECT OF WATER QUALITY ON OVIPOSITION IN *BIOMPHALARIA GLABRATA* (SAY, 1818) (PLANORBIDAE), AND A DESCRIPTION OF THE STAGES OF THE EGG-LAYING PROCESS

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## ABSTRACT

With the overall goal of developing a method to reliably induce oviposition in the freshwater pulmonate *Biomphalaria glabrata*, the effects of water quality on female reproductive physiology were examined. Groups of snails were subjected to controlled experimental conditions consisting of a daily regimen of feeding and water change. After a period of acclimatization, egg mass (EM) output under these conditions was relatively stable, and snails laid a majority (82.5%) of their EM during the initial 4 h following daily water change. When this regimen was perturbed by halting water change for 24 h (dirty-water treatment), EM output was significantly inhibited. When water change was resumed, EM output returned to previous levels within 4 h post-water change (PWC). This dirty-water treatment followed by water change also resulted in a significant increase in mean EM size during the 4 h PWC when compared to controls. To better describe the events preceding egg-laying in *B. glabrata*, we then used these experimental manipulations to induce oviposition in groups of snails, and dissected them during the 4 h following water change. Observations of the reproductive tracts of stimulated snails allowed us to divide the egg-laying process, from ovulation to oviposition, into discrete stages, after de Jong-Brink, Koop, Roos & Bergamin-Sassen (1982). Stage I was characterized by the presence of ova in the hermaphroditic duct and carrefour, and fertilized, packaged eggs in the oviduct and muciparous gland. Stage II was characterized by the presence of packaged eggs in the oöthecal gland embedded in a mucous layer, constituting the egg mass to be laid on the substratum. No packaging events were occurring in the carrefour/albumen gland region during this stage. When snails were dissected immediately after oviposition (Stage III), unpackaged ova were observed in the hermaphroditic duct, carrefour, and oviduct. The mean time it took for snails to reach Stage III was 120 ± 49 min (SD), and this value was statistically different from the mean time to Stages I and II, showing that our induction protocol results in a temporal progression through the egg-laying process. Gonadal oocyte density (oocytes/mm$^2$ of ovotestis) was quantified as a function of these stages of the reproductive cycle, and was found to be significantly lower during Stage II (fully formed egg mass in oöthecal gland) than all other stages examined. Taken together, these results show that female reproductive activity can be experimentally controlled through the manipulation of water quality, and that such a protocol is a valuable tool for addressing specific questions regarding the reproductive physiology of *B. glabrata*. The implications of these results as they pertain to the regulation of female reproductive activity in *B. glabrata* are discussed.

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## INTRODUCTION

Egg-laying behavior in freshwater pulmonates (Basommatophora) is greatly influenced by environmental factors (van der Steen, 1967; ter Maat, Lodder, & Willibrink, 1983; Vianey-Liaud, 1981). Although the signal for ovulation in the Basommatophora is presumed to originate from neuroendocrine cells in the cerebral ganglia (van Minnen, Schallig & Ramkema, 1992), snails must also possess a sensory system capable of transmitting information about the external environment to the reproductive control centers (Janse, van der Plas, van der Roest & van der Wilt, 1983). From past studies it is evident that environmental factors can have both long and short term effects upon the female reproductive behavior of freshwater pulmonates. Numerous studies on the pond snail *Lymnaea stagnalis* have shown that photoperiod (Dogterom, Bohlken & Joosse, 1983), light intensity, food availability, temperature and atmospheric pressure all have long-term effects on reproductive output (Timmermans,

1959; van der Steen, 1967; ter Maat *et al*., 1983; Joosse, 1984). Among the Planorbidae, both temperature (Vianey-Liaud, 1981) and the presence of a conspecific (Vernon, 1995) had positive effects on *Biomphalaria glabrata* egg-laying, while water quality and temperature were found to affect egg output in two species of *Planorbis* (Timmermans, 1959).

Manipulation of the environment can also have more acute, short-term effects on reproductive output. When sexually mature specimens of *L. stagnalis* are kept for several days in dirty water, they cease egg-laying. Transferring these snails to fresh water results in an immediate (within 4 h) resumption of egg-laying (van der Steen, 1967; ter Maat *et al*., 1983). Further studies showed that both the cleanliness and oxygen content of the water had positive effects on this burst of reproductive activity (ter Maat *et al*., 1983). When employed as an experimental tool, this technique allows for reliable control of reproductive activity, and has facilitated studies, almost exclusively in lymnaeid snails, aimed at understanding the biochemical and neuroendocrine events preceding, during, and following egg-laying (Kits, 1980; de Jong-Brink, Bergamin-Sassen, Kuyt & Tewari-Kanhai, 1986; Wijsman & van Wijck-Batenburg, 1987).

Our studies are focused on the reproductive physiology of the planorbid snail, *Biomphalaria glabrata*, due to its medical importance as an intermediate host of the blood fluke *Schistosoma mansoni*, the causative agent of human schistosomiasis (Brown, 1994). Although there have been studies addressing the ecology and biochemistry of *B. glabrata* egg-laying (Vernon, 1995; Bai, Li, Christensen & Yoshino, 1996; Trigwell, Dussart & Vianey-Liaud, 1997), as well as structure/function relationships of different tissues comprising the reproductive tract (de Jong-Brink, de Wit, Kraal & Boer, 1976; de Jong-Brink, Boer & Joosse, 1983), methods to reliably control ovipository activity under laboratory conditions have not been developed. The ability to synchronize egg-laying events in a population of normally non-synchronized snails would greatly facilitate studies on the biochemistry and neuroendocrinology of *B. glabrata* reproduction. Therefore, the aims of the present study were to develop an experimental protocol to reliably induce oviposition in *B. glabrata*, and to use such a method as a tool to describe structural and temporal aspects of the egg-laying process.

## MATERIALS AND METHODS

### *Snail strain and experimental design*

Snail rearing and experimental manipulations were carried out under constant conditions of temperature (26°C), light cycle (12L:12D), and humidity (50%) at the Biotron animal care facility (University of Wisconsin-Madison). All snails used were sexually mature (10–13 mm shell diameter), laboratory-bred specimens of the NMRI strain of *B. glabrata*, initially raised in 38 L aquaria on a diet of green leaf lettuce supplemented with $CaCO_3$ (chalk). In our efforts to achieve reliable induction of *B. glabrata* egg-laying, it was necessary to develop a set of experimental parameters which would result in a predictable initiation of reproductive activity upon stimulation. Preliminary observations (unpublished data) and previous work on *L. stagnalis* (van der Steen, 1967; ter Maat *et al*., 1983) revealed that both frequency of water change and the level of water aeration had dramatic effects on egg-laying. Therefore, experiments that focused on these elements were essential in designing, and eventually arriving at, the specific experimental protocols described here.

In general, following removal from the breeding tanks and size measurements, snails were placed in groups of 10 in plastic beakers containing 650 ml artificial pond water (deionized water plus modified Nolan & Carriker's (1946) salt solution). Groups were fed green leaf lettuce *ad libitum*, and given 2 × 2 cm pieces of styrofoam as their preferred egg-laying substratum (Bai *et al*., 1996). At either 1400 hrs (Experiment I) or 1200 hrs (Experiments II, III) on day 1, the snails were transferred into a clean beaker containing fresh pond water that had been aerated vigorously for 30 sec. Fresh lettuce and egg-laying substrata were added. Reproductive output was quantified during Experiments I and II by collecting egg masses (EM) laid on the substrata, beakers, lettuce, and the snails themselves at the specific times post-water change (PWC) specified below. This regimen of water change, EM collection, and feeding was repeated at the same time daily through to day 6 to allow EM output to stabilize. Experimental manipulations, described below, commenced on day 7 following acclimation.

### *Experiment I: Effect of dirty-water treatment on egg mass output*

In a preliminary experiment, the effect of dirty-water treatment on daily egg mass output was examined. Equilibration was carried out on days 1–6 as described above, with EM being collected and counted at 24 h intervals. On day 7, water change was halted, EM laid in the previous 24 h were collected, and fresh substrata and lettuce were added. During this period of 'dirty-water treatment', the beakers were covered with transparent plexiglas sheets to minimize air/oxygen exchange, leaving an air volume of approximately 150 $cm^3$ above the water in each beaker. On day 8, EM were again collected, and snails were transferred to fresh, aerated pond water as described

above and given fresh egg-laying substrata. At 1400 hrs on day 9, EM were again collected. This experimental protocol was applied to 2 groups of 10 snails, and replicated 7 times.

*Experiment II: Egg output 4 h post-water change, and the effect of 1 and 3 days of dirty-water treatment on egg output*

In order to examine the response to dirty-water treatment and water change in greater detail, a second experiment was carried out in which egg output was quantified during the 4 h immediately following water change, as well as during the next 20 h. Water change was still carried out every 24 h. Therefore, two data points were generated for each day: EM laid during the 4 h following water change (0–4 h PWC), and EM laid during the next 20 h (4–24 h PWC). Both the number of EM and the number of eggs per EM were recorded throughout the duration of the experiment.

As previously described, groups of snails were equilibrated for stable EM output during days 1–6. During this period, EM laid between 0–4 and 4–24 h PWC were collected and counted. On day 7, groups were randomly assigned to one of two test groups: Water change was withheld for half of the groups (dirty-water treatment), while the other half continued to receive water change (control). Feeding was maintained as normal and EM were collected and counted 4 and 24 h later for both groups. On day 8, both groups received a water change, and egg-production was determined for both groups 4 and 24 h PWC. Daily water change was then carried out for all snails on days 8–11 to allow for re-equilibration of the experimental group, and this was followed by a second round of dirty-water treatment, this time for 3 days. Water change was withheld from the experimental group on days 12–14, while daily water change continued for the control group. On day 15, both groups were given a fresh water change, and EM laid 4 and 24 h PWC were collected and counted. Daily water change and egg mass collection were continued through day 16. Each treatment group consisted of 10 groups of 10 snails.

*Experiment III: The effect of dirty-water treatment and water change on the reproductive tract of* B. glabrata

In a third experiment, the effects of dirty-water treatment and water change on the state of the reproductive tract of *B. glabrata* were examined. Following the same protocol as in Experiment I, groups of snails were subjected to dirty-water treatment for 24 h. Most of the snails were then transferred to fresh, aerated water, although some were left in dirty water to serve as controls (not stimulated, NS). At different times during the 4 h PWC, snails were dissected under non-anaesthetized conditions, and the female reproductive tract, from the seminal vesicle to the uterus, was examined for the presence and location of oocytes, packaged eggs, and/or EM. This process, from ovulation to oviposition, has been shown to occur in discrete stages in *L. stagnalis* (de Jong-Brink *et al*., 1982), and our staging system was adapted from these past studies. We separated the internal events leading up to egg-laying into two discrete stages, using the nomenclature of Plesch, de Jong-Brink & Bier (1971). Briefly, Stage I is characterized by the presence of ovulated oocytes, or ova, in the hermaphroditic duct and carrefour, and eggs individually packaged in perivitelline fluid (PVF) in the oviduct (Fig. 1A). Stage II began when all of the eggs that had been packaged were found in the oöthecal gland near the vagina, and were embedded *en masse* in a mucous layer, or tunica interna (Plesch *et al*., 1971; Fig. 1B). This constitutes the egg mass that will eventually be deposited on the substratum. No PVF coating or packaging events took place in the carrefour region during this stage. We also dissected snails immediately after oviposition (Stage III). To examine temporal aspects of this process, the time that had elapsed between the stimulus (water change) and the dissection was noted.

For a subset of snails known to be in Stages I or II of the reproductive process, the ovotestis (OT) was carefully dissected free of the digestive gland, placed in a drop of pond water on a microscope slide, squashed with a coverslip, and examined with a compound microscope to visualize the location and number of mature oocytes in the OT. Ovotestes also were examined from snails immediately after oviposition (Stage III), and from snails left in dirty water (not stimulated, NS). Images of 3–5 fields of view (0.54 $mm^2$ in size) per OT were scanned and loaded into the Picture It!® software package (Microsoft, Pulman, WA, USA), and the number of visible (40–50 μm in diameter) oocytes/field of view were manually counted. Three to six OT's were examined per stage.

*Statistical analyses*

Daily egg output data from Experiment I were expressed as mean EM laid/snail/day ± 1 standard deviation (SD) and subjected to a repeated measures ANOVA for within subject variation using the Statistical Analysis Systems Software (SAS Institute Inc., Cary, NC, USA). Only data from days 4–9 were subjected to the analysis, although all data points are represented in the results (Fig. 2). Post-hoc multiple means comparisons tests for differences between days were performed using Tukey's Honest Significant Difference test (Zolman, 1993).

For Experiment II, data from the 0–4 and 4–24 h PWC time points were expressed as mean EM laid/snail/h ± SD for all 10 beakers of snails in each treatment group. Differences between control and treatment groups were analyzed using Student's t-test, and only pre-planned comparisons of the days before, during, and after dirty-water treatment were performed to avoid excessive Type I error. The mean number of eggs/EM was analyzed in a similar fashion. Oocyte density data were expressed as oocytes/$mm^2$ ± SD for each field of view, segregated into one of 3 egg-laying stages, and analyzed along with controls using a Completely Randomized Design with subsampling, with snail as the whole plot and field of

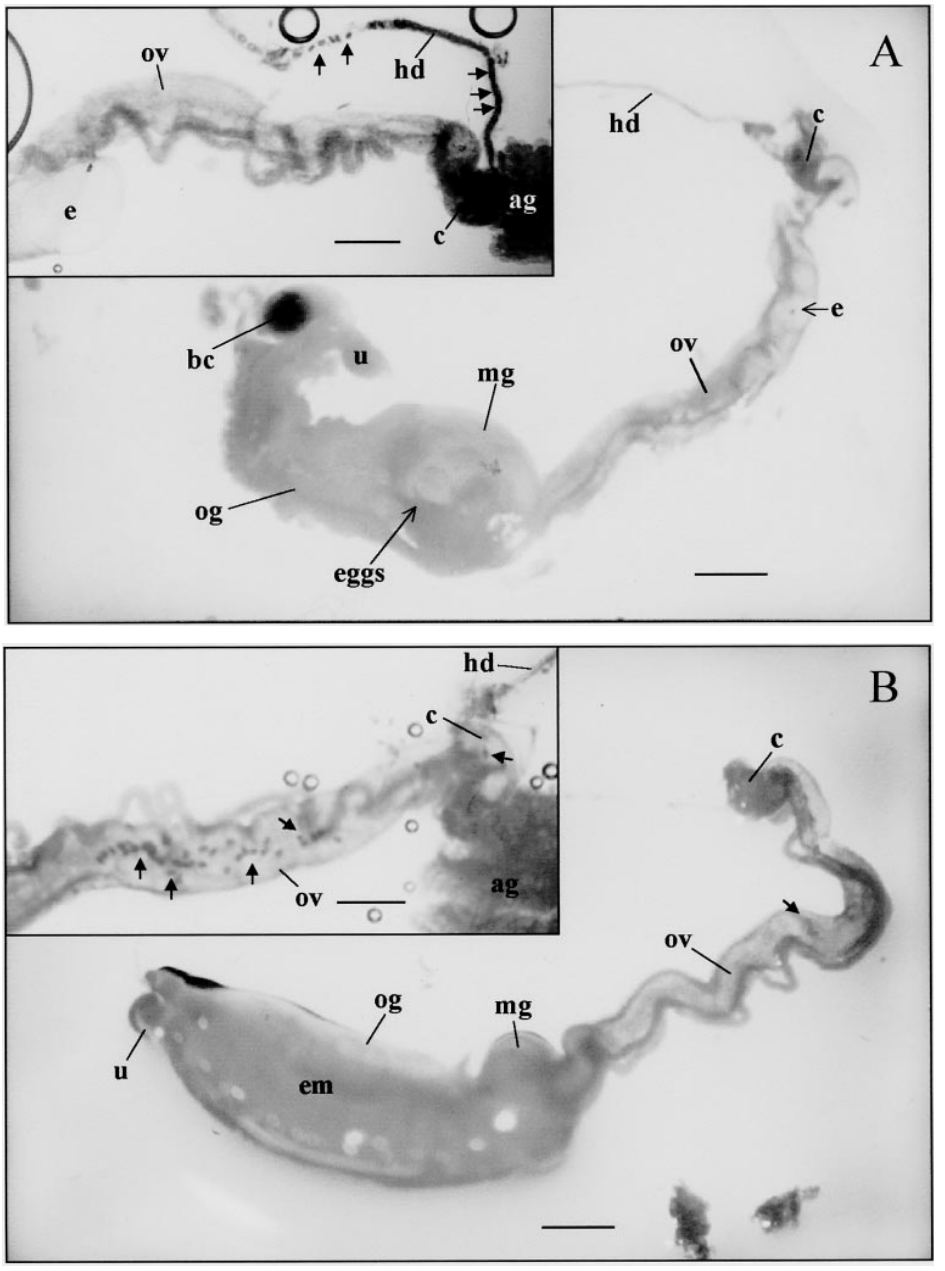


**Figure 1.** Photomicrographs of dissected reproductive tracts of *B. glabrata* illustrating the two internal stages of the egg-laying cycle. **A.** Stage I. Individually packaged eggs (e) can be seen in the oviduct (ov), and these eggs congregate in the region of the muciparous gland (mg). Note that the albumen gland has been removed. Scale bar = 2 mm. Inset: ovulated oocytes are visible in the hermaphroditic duct (hd) and carrefour (c), and an individually packaged egg (e) is visible in the oviduct (ov) (the oviduct was broken, releasing the egg). Scale bar = 1 mm. **B.** Stage II. The fully formed egg mass (em) can be seen in the oöthecal gland (og) near the uterus (u), and naked oocytes (arrow) can be seen in the oviduct (ov). Note that the albumen gland has been removed. Scale bar = 2 mm. Inset: Again, naked oocytes (arrows) can be seen in the carrefour (c) and oviduct (ov). Scale bar = 1 mm. Abbreviations: ag, albumen gland; bc, bursa copulatrix; c, carrefour; e, individually packaged egg; em, egg mass; hd, hermaphroditic duct; mg, muciparous gland; og, oöthecal gland; ov, oviduct; u, uterus.

view as the subplot (Zolman, 1993). Tukey's multiple comparison post-test was used to determine differences between means. All differences were deemed significant at $P \leq 0.05$.

## RESULTS

*Experiment I:*

For all groups in Experiment I, no EM were laid on day 1, possibly due to the initial stress of handling and measuring the snails and/or the differences in environmental conditions between the breeding tanks and plastic beakers. However, by day 3 all groups were consistently laying EM, and by day 4 EM output was quite stable (Fig. 2). Repeated measures ANOVA revealed significant within-subject variation in egg output during days 4–9 ($P < 0.0001$). During the period of no water change, EM output decreased relative to the previous day's output, from 0.508 ± 0.256 (day 6–7) to 0.023 ± 0.063 EM/snail/day (dirty-water treatment, day 7–8). Multiple comparisons revealed that output for day 7–8 was significantly lower than any other day analyzed. A day after transfer to fresh water, mean EM output significantly increased to 1.4 ± 0.38 EM/snail/day, and post-tests revealed that this value was significantly higher than all other days analyzed (Fig. 2).

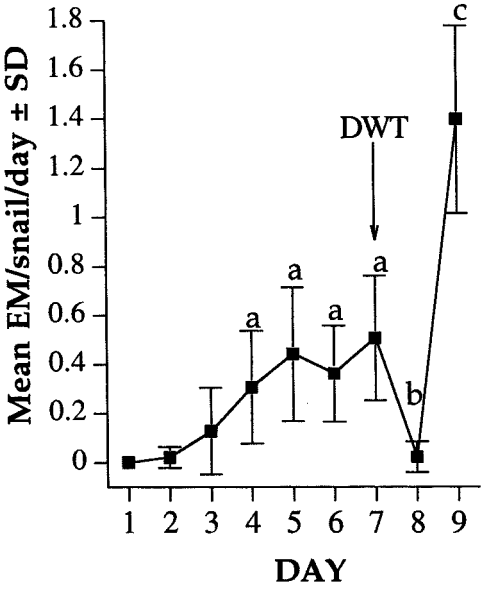


**Figure 2.** Egg-laying rate (mean EM/snail/day ± SD) for groups of snails in Experiment I showing the effect of dirty-water treatment and subsequent water change on egg mass (EM) output. After EM collection, water change was carried out on all days of the experiment except for day 7 (arrow, dirty-water treatment, DWT). Common letters indicate days not significantly different from each other ($P > 0.05$) using repeated measures ANOVA followed by Tukey's Honest Significant Difference test (days 4–9 only).

*Experiment II:*

In Experiment II, snails began to lay EM by day 2, and output was stable by day 5. Egg output information for the control group alone is summarized in Table 1. The most striking feature of the observed egg-laying pattern is that 82.5% of the total EM laid by the control group during the experiment were laid during the initial 4 h following daily water change. During the subsequent 20 h (4–24 h PWC), egg output was significantly lower, with a mean egg-laying rate of 0.005 ± 0.002 EM/snail/h.

The effects of the 1 and 3 day dirty-water treatments and subsequent water change on egg mass production are represented separately in Figs. 3A (Days 6–9) and 3B (Days 11–16). During the 4 h following the first dirty-water treatment (Day 7) the treated group (no water change) laid no EM, and this was significantly different from controls ($P < 0.0001$). During the next 20 h egg output for the two groups was not significantly different. Upon transfer to clean water on Day 8, the egg-laying rate in the treated group returned to control levels at the 4 h PWC time point, with treated snails laying 0.184 ± 0.052 EM/snail/h compared to the control snail rate of 0.184 ± 0.045 EM/snail/h (Fig. 3A). Dirty-water treatment also had significant effects on the number of eggs per EM

**Table 1.** Egg-laying response of control *B. glabrata* snails (Exp. II) subjected to regular water changes at 24 h intervals: A comparison of the 0–4 h and the 4–24 h post-water change (PWC) time points.

| | 0–4 h PWC | 4–24 h PWC |
|---|---|---|
| Mean EM/snail/h | 0.135 ± 0.022 | 0.005 ± 0.002 |
| Mean eggs/EM | 34.9 ± 6.8 | 35.4 ± 8.4 |
| Total EM | 758 | 161 |
| % of total EM | 82.5 | 17.5 |
| Total eggs | 26,853 | 5,815 |

Note: EM/snail/h and eggs/EM data are reported as the mean of 10 groups of snails averaged over days 4–16 ± SD. Likewise, total EM and egg values represent the cumulative output for days 4–16. Abbreviations: PWC = Post-water change, EM = egg mass.

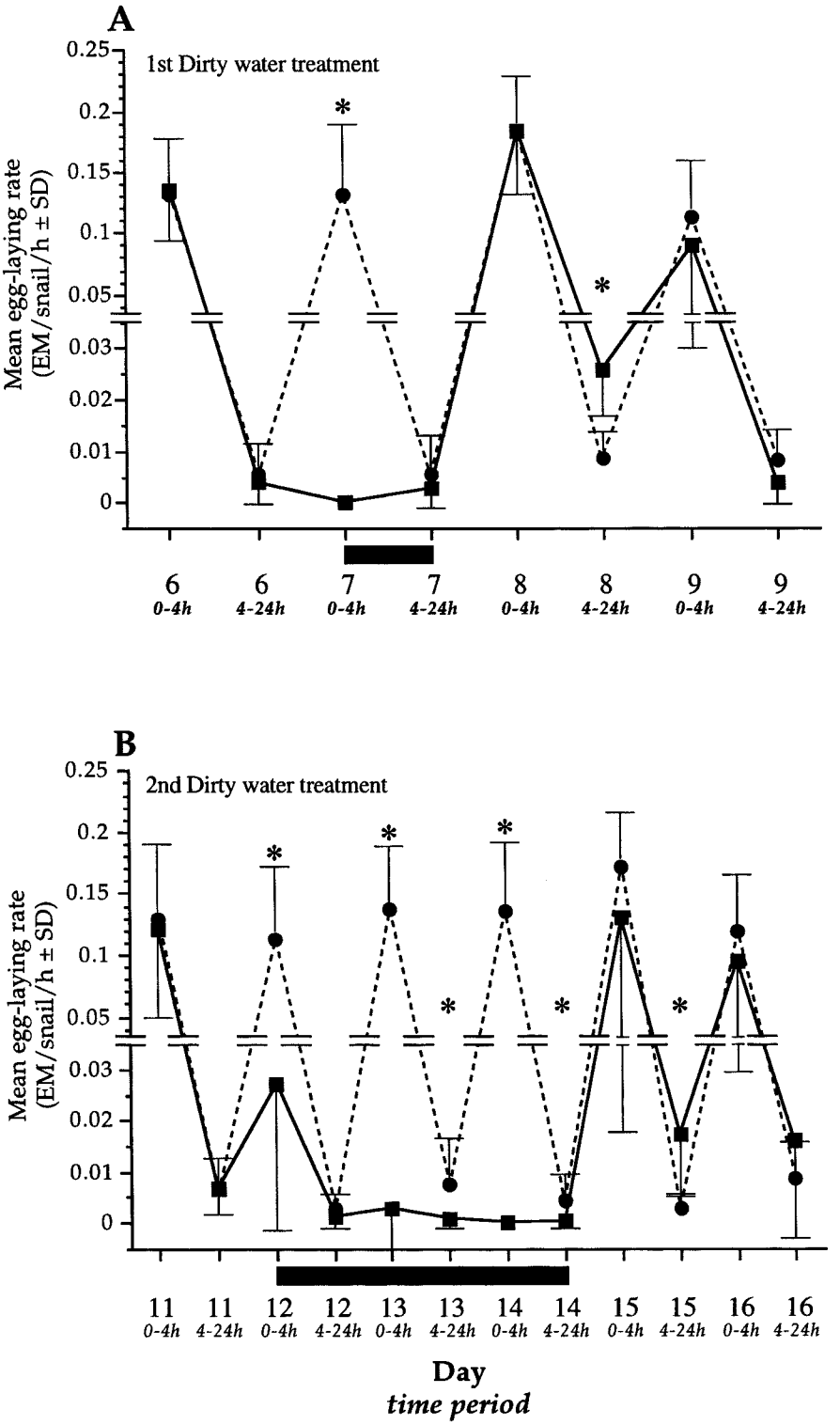

**Figure 3.** Effect of dirty-water treatment on egg-laying rate (mean EM/snail/h ± SD) of control (**---●---**) and treated (**—■—**) groups of snails for the 1 day (**A**) and 3 day (**B**) dirty-water treatments in Experiment II. Water change was carried out every 24 h, and egg-laying rate is divided into two time periods: the 4 h following water change (0–4 h post-water change (PWC)), and the subsequent 20 hours before the next water change (4–24 h PWC). The black bar underscores the time periods during which water change was halted for the treatment group (dirty-water treatment). Asterisks indicate time periods when egg-laying rates of control and treatment groups differed significantly (Student's t-test, $P \leq 0.05$). Both graphs illustrate the burst of reproductive activity observed 4 h PWC compared to the next 20 h (Note the break in scale. Also, Fig. 3A contains data from 5 days, while graph Fig. 3B contains data from 7 days, resulting in a more compressed abcissa in 3B). **A.** Mean egg-laying rate for the treatment group was significantly lower during the period of no water change (day 7, 0–4 h), and this rate returned to control levels upon resumption of water change (Day 8, 0–4 h). Also, treatment groups laid egg masses at a significantly higher rate during the next 20 h (Day 8, 4–24 h). **B.** Similar results were obtained during the longer period of dirty-water treatment, showing that the treatment can inhibit egg-laying for up to 3 days. Egg-laying can still be rescued upon resumption of water change (Day 15, 0–4 h), and again results in a significantly higher egg-laying rate than controls during the next 20 h (Day 15, 4–24 h), despite the negative effects observed on 2 of the 10 treatment groups (see text).

laid during the 4 h period following resumption of water change. EM laid by the treatment group during this period (Day 8, 4 h PWC) contained significantly more eggs/EM than controls ($P = 0.021$, Fig. 4). During the next 20 hours, the treated group laid EM at a significantly higher rate than controls ($P < 0.001$, Fig. 3A), and these EM were found to contain significantly fewer eggs than controls ($P = 0.049$, Fig. 4). These data show that 24 h of dirty-water treatment abolishes the burst of reproductive activity normally seen 4 h PWC, but such activity is rescued (within 4 h) by transfer to clean water. Rescue results in increased EM size during the 4 h following water change, and a significant increase in reproductive activity during the subsequent 20 hours. By the 4 h PWC time point on day 9, egg output for the treated group, both in terms of EM/snail/h and eggs/EM, returned to control levels.

When water change was again halted on day 12, the treated group again laid significantly fewer EM than controls ($P < 0.001$) during the 4 h after dirty-water treatment, and egg output continued to be significantly less in the treated groups than controls on days 13 and 14 at the 4 h PWC time point ($P < 0.0001$ for both days, Fig. 3B). Upon transfer to fresh water on day 15, egg-laying rate in the treatment group returned to control levels 4 h PWC, with treated snails laying 0.131 ± 0.114 EM/snail/h and controls laying 0.171 ± 0.045 EM/snail/h (Fig. 3B). Egg output in the treatment group was again significantly higher than controls during the next 20 h ($P = 0.002$). However, there was no significant difference in the number of eggs/EM between the control and treatment groups at the 4 h PWC time point on day 14, although the number of eggs/EM was significantly lower in the treatment group during the subsequent 20 h ($P = 0.016$, Fig. 4). Worthy of note is that following this three day dirty-water treatment, two of the 10 experimental groups failed to lay any EM at the 0–4 and 4–24 h PWC time points. For these groups only, no excrement was observed in the beakers following water change, suggesting that during the prolonged dirty-water treatment some snail groups apparently stopped feeding. This cessation of

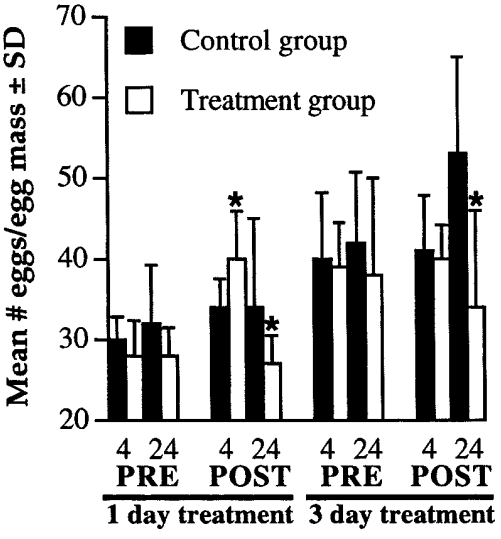


**Figure 4.** The effect of 1 and 3 days of dirty-water treatment on mean egg mass (EM) size before (PRE) and after (POST) each cycle of dirty-water treatment. For each day, mean EM sizes are represented at both the 0–4 h post-water change (PWC) time points ('4') and 4–24 h PWC time points ('24'). Treatment group means with an asterisk (*) are significantly different from controls as determined by Student's t-test ($P \leq 0.05$, N = 10 for each group).

feeding behavior was not observed when these snails were subjected to only 24 h of dirty-water treatment (in either Experiment I or II).

*Experiment III: Effect of water quality on the progression of egg and egg-mass formation:*

As described above, dirty-water treatment can be used as a tool to halt egg-laying for a period of time, and it can be quickly resumed by transferring snails to fresh water. We examined the reproductive tracts of snails under dirty water conditions (1 day of dirty-water treatment, NS), and also observed changes that occurred upon transfer to fresh water.

Reproductive tracts of all control snails that were left in dirty water (NS) were completely empty of ovulated oocytes (ova), packaged eggs, or EM, suggesting that dirty-water treatment completely halts the egg-laying process. When snails were dissected during the first 60 min after transfer to fresh water, the reproductive tracts were generally devoid of any signs that the egg-laying process had begun. However, when snails were dissected 60–120 minutes PWC, both Stages I and II of the reproductive process were evident. The mean time to Stage I was 87 ± 22 min, which was not significantly sooner than Stage II (mean time = 99 ± 20 min), suggesting that these two stages occur in rapid succession (Table 2). Stage I was exactly as described in methods, and as long as ova were being packaged in the carrefour, snails were considered to be in this stage. During Stage II, in addition to the fully formed EM present in the oöthecal gland, the reproductive tract also contained many unpackaged ova in the carrefour and oviduct. In certain cases, the number of these 'abandoned' ova exceeded the number that had been packaged and embedded in the egg mass (Fig. 1B). Stages I and II were the only discrete internal stages of the egg-laying process that we observed. The mean time to oviposition (Stage III) was 120 ± 49 min, and statistical analyses revealed that the mean time to this stage was significantly longer than both Stages I and II (Table 2). When snails were dissected during this stage, unpackaged ova were still observed in the hermaphroditic duct, carrefour, and oviduct (not shown), although generally fewer than seen during Stage II. The egg packaging and egg mass formation events characteristic of Stages I and II were not observed. If dissections were made longer than 15 minutes after oviposition, the reproductive tract was found to be completely empty of ova, eggs, and EM (data not shown).

When the gonads of snails in different stages of the reproductive process were examined, we observed no gross morphological differences between OT's dissected from control and Stage I–III snails. Oocytes were evenly dispersed throughout all of the gonads examined, both in the acini as well as in the OT lumen. However, statistical comparisons of mean oocyte density for control and staged snails revealed that oocyte density did vary significantly with stage (one-way ANOVA, $P = 0.02$), and that Stage II (EM fully formed) gonads had the lowest oocyte density (Tukey's HSD test, $P < 0.05$, Fig. 5).

**Table 2.** One day of dirty-water treatment followed by water change induces a temporal progression through the stages of the egg-laying process of *B. glabrata*. Mean (SD) and range (minimum-maximum) values of the time (in min) that elapsed between the resumption of water change and the observation of Stages I–III. N = 22 for Stage I, 35 for Stage II, and 20 for Stage III.

| | STAGE | | |
|---|---|---|---|
| | I | II | III |
| | Mean 87 | 99 | 120 |
| (SD) | (22)[a] | (20)[a] | (49)[b] |
| Range | 47–135 | 68–158 | 34–235 |

Note: Means with the same letter are not significantly different as determined by one-way ANOVA and Tukey's HSD test.

## DISCUSSION

In this study, our aim was to develop an experimental protocol to control reliably the female reproductive activity of *Biomphalaria glabrata*. While 'spontaneous' oviposition can be observed in freshwater snails (Wijsman and van Wijck-Batenburg, 1987), it happens at such a low frequency that it is not suitable for large-scale experimental manipulations. Using a set of controlled experimental parameters, we were able to effectively control *B. glabrata* reproductive output, and subsequently predict when groups of snails were most likely to be in the process of ovulating, packaging, or laying, eggs. This allowed us to describe these processes on a structural level, and better understand their regulation.

The effect of water change on oviposition has been reported in a few species of freshwater pulmonates (Timmermans, 1959), although it

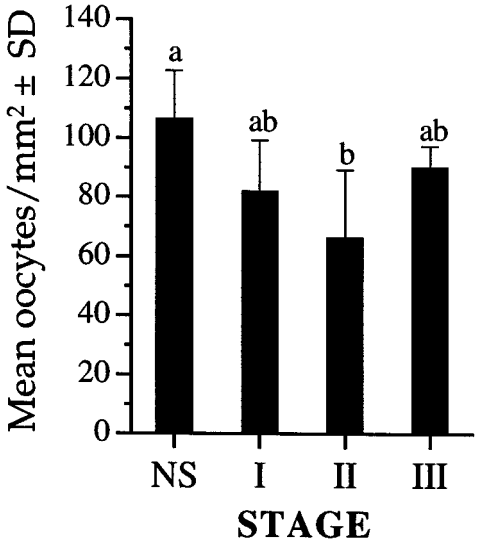


**Figure 5.** Mean oocyte density in the ovotestis (OT) from dirty water control (NS) snails and those known to be in different stages of the egg-laying cycle. Data are expressed as mean number of oocytes/mm$^2$ of OT ± SD. Groups with the same letter are not significantly different ($P > 0.05$) as determined by one-way ANOVA and Tukey's HSD test. Analysis revealed that the mean oocyte density of Stage II (EM packaged in oöthecal gland) gonads was significantly lower than all other stages examined. Mean density returned to control levels by Stage III (immediately after oviposition). Abbreviations: NS, not stimulated (dirty water control).

has been exploited as an experimental tool only in *L. stagnalis*. Under the experimental conditions described, *B. glabrata* oviposition was found to be highly dependent upon water change. One of the most striking examples of this dependence was the fact that in Experiment II, control snails laid over 80% of their total EM during the first 4 h following daily water change (Table 1). This synchronization was apparent as soon as the snails began to lay eggs, and persisted throughout the experiment. This is consistent with reports in *L. stagnalis*, where, under similar conditions, 70% of the EM were laid during the 3 h period following water change (van der Steen, 1967).

In this light, it may not be surprising that *cessation* of water change (i.e., dirty-water treatment), even for a 24 h period, had such potent effects on oviposition in this study. In both Experiments I and II, very rarely did snails lay eggs during the period of dirty-water treatment, suggesting that chemical substances present in the environment, possibly of snail origin, and/or lowered water oxygen tension, may provide a signal that results in the inhibition of ovipository activity. The effect of this signal was not chronic, since transferring snails to fresh water quickly removed its inhibitory activity. However, when the duration of dirty-water treatment was extended to 3 days, we observed more long-term effects on snail reproduction, in that 2 of the 10 treatment groups took 24 h to resume normal egg-laying behavior. Such a difference in response to dirty-water treatment suggests the presence of diverse mechanisms regulating reproductive activity in response to different environmental conditions. One can imagine that the responses to dirty water described in this study are very similar to the reduced survivorship and fecundity observed when snails are raised at high densities, the so-called 'crowding effect' (Chaudhry, 1987; Loreau & Baluku, 1987). This response to water quality could be highly adaptive considering that the normal habitats of freshwater pulmonates, like *B. glabrata*, are often subjected to drastic changes in moisture, food availability, temperature, and light (Mozley, 1954). Snails populations are thought to decrease during unfavorable periods (e.g., drought), and to respond to the amelioration of conditions with high reproductive output. van der Steen (1967) has proposed an additional hypothesis for the clean water response in *L. stagnalis*. In their native habitats, these snails prefer to oviposit directly on the leaves of water-dwelling plants, and photosynthetic oxygen emitted from the leaf surface could act as a reproductive stimulant. Indeed, oxygen content is known to be an important component of the stimulatory effect of fresh water on *L. stagnalis* egg-laying (ter Maat *et al*., 1983), and oxygen tension has been shown to have effects on snail locomotory behavior (Janse, 1981) as well as behaviors related to reproduction (Janse *et al*., 1983). Obviously, freshwater pulmonates have evolved systems that are capable of sensing changes in the environment, and translating them into the regulation of diverse physiological processes.

Another major effect of dirty-water treatment and water change was the increase in the number of eggs/EM produced during the first 4 h following the resumption of water change in Experiment II. This increase in EM size observed after dirty-water treatment is consistent with previous work in *L. stagnalis*, where EM size was found to be positively correlated with the time interval between egg-laying

events (van der Steen, 1967; ter Maat *et al*., 1983). One possible explanation for these results is that oocytes build up in the OT during dirty-water treatment, and when snails are transferred to fresh water there are more oocytes available for packaging into the egg mass. However, this seems unlikely in view of our observations of unpackaged ova throughout the reproductive tract during stages in which the egg mass has been fully formed (Stage II) or immediately after it has been laid (Stage III; see Figs. 1A and 1B). A more likely explanation of the increase in EM size is that the number of eggs that are packaged into an EM is determined by the physiological state of the albumen gland (AG). Secretions from this gland coat each fertilized egg with a discrete amount of perivitelline fluid (PVF), providing the developing embryo with protein and galactogen (de Jong-Brink *et al*., 1983). It is possible that when AG stores have been exhausted, or when some regulatory signal causes the AG to cease secreting fluid, the ovulated oocytes that remain in the hermaphroditic duct and carrefour simply pass the AG and proceed up the oviduct to be resorbed in the bursa copulatrix (de Jong-Brink *et al*., 1983). In the case when snails have not been laying eggs due to dirty-water treatment, the AG could continue to synthesize and store PVF, and when water change is resumed there may be more of this fluid in the gland available for coating additional eggs (Wijsman & van Wijck-Batenburg, 1987).

The quantification of gonadal oocyte density during the egg-laying cycle revealed that although mean oocyte density was significantly lower than controls during Stage II, large fluctuations in oocyte density did not occur. Our method of quantification focused on oocytes of the same size (40–50 μm) that are ovulated into the hermaphroditic duct during Stage I, and, according to light and electron microscopic descriptions of the ovotestis of *B. glabrata* (de Jong-Brink *et al*., 1976), this size range fits into the most mature class of oocytes. In the Basommatophora, oogenesis is known to be at least partly under the control of hormones secreted by the endocrine dorsal bodies (Geraerts & Joosse, 1975; Saleuddin, Kunigelis, Schollen, Breckenridge & Miksys, 1983), and both the secretion and the effects of these hormones on the ovotestis are thought to be long term, (de Jong-Brink & Goldschmeding, 1983). Such a consistent release of hormone could cause oocyte maturation to occur at a rate that assures the presence of a large number of mature oocytes in the ovotestis, even though only a small percentage of these oocytes are actually ovulated during an egg-laying event. This could be due to an additional step in the oocyte maturation process that would prepare a certain subset of oocytes for ovulation. Another possible explanation would be that a large majority of the mature oocytes counted here are degenerative, having missed the proper time window to be successfully ovulated (de Jong-Brink *et al*., 1983). Regardless of the reasons for such consistent oocyte numbers during the egg-laying cycle, these data, paired with the observations of unpackaged oocytes in the oviduct following EM formation described above, suggest that maturation and ovulation of oocytes is less tightly regulated than is the production of fully packaged eggs.

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