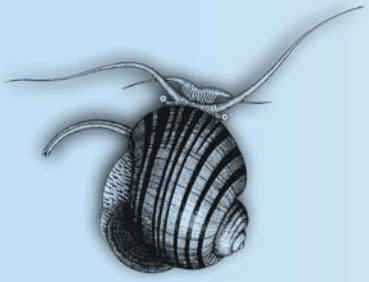

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THE EFFECT OF LIGHT ON INDUCED EGG LAYING IN THE SIMULTANEOUS HERMAPHRODITE *LYMNAEA STAGNALIS*

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ABSTRACT

Reproduction is influenced by many external factors. For egg laying of pond snails, one important trigger is the transfer from dirty, oxygen-poor water to clean, oxygen-rich water. This response is due to the combined effects of elevated oxygen level, chemical water composition and clean substrate. Whether this clean-water stimulus (CWS) resembles the natural egg-laying process has remained untested. Given that the response relies heavily on a pretreatment that suppresses egg laying, the animal's internal state is clearly important. Egg laying is known to be influenced by day length, hence external factors signifying time of day or season may be involved. We here study the effect of light on the CWS in the freshwater pulmonate *Lymnaea stagnalis*. Clean water was more effective in inducing oviposition in the light than during darkness, irrespective of the presence of eyes. Thus, light has a profound influence on egg laying, which is most likely mediated by nonocular photoreceptors. We show that more eggs are laid during the day than during the night in wild-caught animals kept outside, which indicates that the effect of light on CWS-induced egg laying is relevant for the induction of egg laying under natural conditions.

INTRODUCTION

Together with environmental and social factors, circadian and seasonal factors play an essential role in regulating reproduction in animals (e.g. Goldman *et al.*, 2004). Such factors can interact with each other as well as with the hormonal and neural mechanisms that regulate reproduction (Visser *et al.*, 2010). Clearly, one important component of circadian and seasonal patterns is light. In order to study the influence of the perception of light on the regulation of reproduction, detailed knowledge about the underlying regulatory mechanism is essential. Only such an integrative approach will be able to tell us how external factors, like light, interact with the internal ones that regulate reproductive output (Visser *et al.*, 2010).

A simultaneous hermaphrodite in which we understand the regulation of female reproduction in detail is the freshwater pulmonate snail *Lymnaea stagnalis* (reviewed by Koene, 2010). Egg laying is controlled by a bilateral group of neurons in the cerebral ganglia, the caudo-dorsal cells (CDCs). These neurons are electrically coupled and release a number of different peptides, including the egg-laying hormone CDCH, during periodic bouts of synchronous bursting activity *in vitro* (De Vlieger *et al.*, 1980; Geraerts & Hogenes, 1985; Jimenez *et al.*, 2004). *In vivo* recordings and stimulations of the CDCs have confirmed that these cells control egg laying (Ter Maat *et al.*, 1989). Additionally, when the hormone, which is normally released during the long-lasting discharge of the CDCs, is experimentally introduced into the blood, egg-laying behaviour follows (Ter Maat *et al.*, 1989).

Like most Basommatophora, *L. stagnalis* lays its eggs in egg masses that it fixes to the substrate. The behavioural phases of egg laying of *L. stagnalis* have been described in detail (Goldschmeding, Wilbrink & Ter Maat, 1983; Ferguson *et al.*, 1993). In brief, during the resting phase the animal stops locomoting and pulls the shell forward over the tentacles. During this phase CDCH is released (Hermann *et al.*, 1997). This is followed by a turning phase in which the animal turns its shell back and forth by 90° and rasps the surface. During this phase the animal cleans the substrate for proper attachment of the egg mass (Ter Maat *et al.*, 1989). The egg mass emerges from the female gonopore and is fixed to the substrate in the oviposition phase and is subsequently checked during the inspection phase, after which the animal leaves. Once a CDC discharge has started, releasing the egg-laying hormones, it takes the animal around 2 h to build the egg mass internally and subsequently fix it to the substrate (Ter Maat *et al.*, 1989).

Egg masses can be laid at a frequency of more than one mass per week, and such masses typically contain between 50 and 150 eggs depending on the individual's body size (Koene, Montagne-Wajer & Ter Maat, 2007) as well as the time since

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the last egg mass was laid (Ter Maat, Lodder & Wilbrink, 1983). Decreases in egg laying are caused by short light periods (Bohlken & Joosse, 1982; Ter Maat *et al*., 2007), frequent mating (Van Duivenboden, Pieneman & Ter Maat, 1985; Koene, Brouwer & Hoffer, 2009) and low temperatures (Vianey-Liaud, 1981; Dogterom *et al*., 1984). Egg laying completely ceases when food supply is very low (Bohlken *et al*., 1986; Ter Maat *et al*., 2007), at old age (Janse, Wildering & Popelier, 1989) and in dirty water (Ter Maat *et al*., 1983). In the latter case, transfer of a snail from dirty to clean water, known as the clean-water stimulus (CWS), reliably elicits egg laying within 2 h (Ter Maat *et al*., 1983). This phenomenon—that egg laying of freshwater pulmonates can be induced by exposing them to a plentiful supply of clean, fresh water—has been known for over a century (e.g. Linville, 1900). The factors involved in this stimulation have been studied in detail by Van Nieuwenhoven & Lever (1946), Timmermans (1959) and Ter Maat *et al*. (1983). The response was found to be due to the combined effects of elevated oxygen content, chemical composition of the water and a clean substrate. In laboratory studies this CWS has been used as a means to induce egg laying reliably, for instance to study the egg-laying process of *Biomphalaria glabrata* (Boyle & Yoshino, 2000), *Bulinus octoploides* (Rudolph & White, 1979) and *Ancylus fluviatilis* (Bondesen, 1950) or egg-laying behaviour in *L. stagnalis* (Ter Maat *et al*., 1989). The importance of the CWS for the natural egg-laying process has remained untested. In view of the fact that the response relies heavily on a pretreatment in which egg laying is suppressed, the internal state of the animal is clearly important. In addition, other external factors signifying time of day or season are likely to be involved. These factors may simply add on to the ones described for the CWS, but they may also have a gating function. In this study we tested the effect of light on egg laying elicited by clean water in *L. stagnalis*.

In addition to eyes, *L. stagnalis* also possesses nonocular photoreceptors and therefore we also investigated whether presence of the eyes is critical in mediating the effect of light on egg laying. Previous work has already shown that nonocular photoreceptors are important for the escape response elicited by shadow and probably also the learning of this response (Sunada *et al*., 2010a, b). Besides a role in predator avoidance, nonocular photoreception plays a role in the animal's orientation in its environment, for which the eyes become essential only under low light conditions (Van Duivenboden, 1982).

MATERIAL AND METHODS

We used adult snails (shell lengths $\pm$ 3 cm) obtained from the culturing facility at VU University, kept under a standard 12:12 L:D cycle in flowing low-copper water at 20°C. The animals were fed lettuce *ad libitum* and were housed individually in perforated plastic jars (with a water volume of 460 ml) equipped with a cover that prevents escape of the animal yet permits oxygen exchange with the air. The jars were placed in two large tanks with continuous water circulation and partial water refreshment (see details in Van der Steen, 1967). With one snail inside, the oxygen content of the perforated jars is near saturation and in the closed jars it is about 5 mg/l (Mooij-Vogelaar, Jager & Van der Steen, 1975). Each tank contained 96 randomly chosen animals. The light fixtures were mounted inside the cover to enable control over the lighting conditions using a natural-light spectrum. The room in which the tanks were placed was lit by a safelight (620 and 750 nm). The sensitivity of this species' photoreceptors is very poor above 640 nm (Sakakibara *et al*., 2005). The animals were distributed randomly over the two tanks, which each had a different light regime. In one tank, the lights came on at 10:00 h, whereas in the second tank they were switched off at 10:00 h; in both tanks the lights switched again after 12 h.

Sixteen days after they were put in the tanks, the animals were placed (using the same water) in closed jars (i.e. not perforated, but the same size and shape, filled with 460 ml of low-Cu water) in the same tank to ensure temperature control for 8 d, during which they received food daily. Pots again had open lids preventing escape of the animal yet permitting oxygen exchange with the air (Ter Maat *et al*., 1983). At day 24 the animals were transferred to clean, perforated jars, placed in the same tank. Under normal daylight conditions, this water change has previously been shown to be a stimulus for egg laying—the CWS (Ter Maat *et al*., 1983). We also know that these animals are not pheromonally induced to lay eggs when they share the same water with animals that are laying eggs (J.M. Koene, unpubl.).

In order to test the effect of time of day, in the first experiment, for the four groups in which lights went off at 10:00 the CWS was delivered at 8:00, 11:00, 14:00 and 17:00 h. In other words, the series started 2 h before lights-off. The second set of groups received the first CWS during darkness, i.e. 2 h before lights-on, and the second, third and fourth CWS at 1, 4 and 7 h after lights-on. Egg laying of all individuals was checked 3.5 h following the CWS.

In the second experiment, we tested the effect of light perception via the eyes on induced egg laying using a different set of animals. Half of the animals had their eyes removed as described by Van Duivenboden (1982). In brief, the animals were anaesthetized by injecting 2 ml of 50 mM $MgCI_2$ into the foot. The eyes were carefully removed under a dissection microscope using a pair of fine forceps and small surgical scissors. The animals recovered from this surgery within 4 h. That all operations had been successful was determined afterwards by carefully examining the skin at the base of the tentacles. The pretreatment described above was repeated up until the day of the experiment (day 24). Sixteen days postoperation, the animals were placed in the tank's water in closed jars for 8 d (as described above). Then all 177 animals received the CWS at 165 min after the light change (12:45 h). Half of each group consisted of animals without eyes, the other half was unoperated. In addition, half of the animals of each tank were transferred to clean jars in the other tank, and the other half was transferred to clean jars in their own tank. This created eight treatments: animals transferred from dark to dark with (n = 22) and without eyes (n = 21), from dark to light (n = 22 and 23), from light to light (n = 23 and 22) and from light to dark (n = 23 and 21). Care was taken not to open the lids of the two tanks at the same time. As in the previous experiment, egg laying was checked 3.5 h following the CWS.

To examine spontaneous egg laying we recorded the number of egg masses of 42 isolated laboratory-raised animals for 96 h. These animals were kept under a 12:12 L:D cycle and egg masses were collected at the end of the light and the dark period. In addition, wild-caught animals were placed in snail-breeding tanks located outside, exposed to natural light and temperature conditions. The pattern of egg laying in these wild-caught snails was assessed by observing two groups of snails, caught in the Eempolder (The Netherlands) in June and July, respectively, for 156 and 216 h. In Amsterdam, in June/July the sun rises and sets at 5:15/5:30 and 22:00/21:45 h, excluding *c*. 1 h of dawn and 1 h of dusk, and the water temperature in the ditches oscillates around 20°C. The snails were fed lettuce *ad libitum* and individually housed in our standard perforated plastic jars of 460 ml in one breeding tank containing flowing low-copper water placed outside 2 d after they were caught. The egg masses were collected at 08:00 and 20:00 h each day, hence during one period darkness prevailed and during the other period it was predominantly light.

RESULTS

Effect of light on stimulated egg laying

The effect of light on egg laying is shown in Figure 1. Overall, with series 1 and 2 combined, the animals receiving the CWS in darkness laid significantly less (19 out of 93; 20.4%) than the animals receiving CWS when it was light (62 out of 95; 65.3%; Fisher's exact test, $P < 0.0001$).

To test the effect on the response variable 'egg laying', we used a nominal logistic model with series and CWS timing as fixed factors. There was no overall difference in responsiveness depending on the timing of the CWS delivery ($\chi^2 = 1.44$, df = 3, $P = 0.69$). There was a difference between the two series ($\chi^2 = 4.087$, df = 1, $P = 0.043$), indicating that the CWS is most effective during the day. In addition, the interaction between the CWS timing and the series was highly significant ($\chi^2 = 31.93$, df = 3, $P < 0.0001$). Clearly, this was caused by the CWS that was given 2 h prior to the light switching on or off. As can be seen in Figure 1, the first time point of each series deviated from the other time points. For the series where the lights were switched on, this difference can be seen by comparing the first black data point with the other three in Figure 1 (i.e. 1, 4 and 7 h). Likewise, for the series where the lights were switched off, this can be seen by comparing the first white data point with the other three (i.e. 13, 16 and 19 h). These results indicate that there is no large variation during the light period and that, once the lights are off, the response is also relatively constant. To test this we compared the data by grouping them according to whether the lights were on or off during the CWS (i.e. this grouping was done across the two series). As expected, there is no significant differences between the four different time points in lights-on, i.e. the time points in the white area of Figure 1 ($\chi^2 = 3.99$, df = 3, $P = 0.26$). Neither do we find a difference between the four time points in lights off, i.e. the grey area in Figure 1 ($\chi^2 = 0.95$, df = 3, $P = 0.81$).

Of the animals receiving the CWS during lights-on at 2 h before the start of the dark period, 19 out of 24 responded with egg laying within 3.5 h. After 6 h of darkness following the checking of egg laying, none of the five remaining animals that had not responded earlier had laid eggs. By contrast, of the 19 nonresponding animals in the group of 24 that was CWS-stimulated at 2 h before lights-on, 10 laid eggs during the 6-h period of light, following the initial 3.5-h observation period. This residual response is significantly different between the two groups (Fisher's exact test: $P < 0.05$).

In summary, there is a clear effect of light on CWS-induced egg laying. Clearly, the response is larger when it is light than when it is dark.

Light perception and induced egg laying

To investigate the role of light perception in the CWS, the CWS was delivered 2.5 h after a change from dark to light or vice versa. Four combinations were made of the light conditions from which the animals came and the light conditions in which they received the CWS. Animals could come from lights-on or lights-off (original light condition) and receive the CWS in lights-on or lights-off (CWS light condition), making a total of four possible transitions. In addition, both animals with and without eyes were subjected to each of the four transitions.

To test the effect on the response variable 'egg laying', we used a nominal logistic model with the presence/absence of eyes, original and CWS light conditions as the three fixed factors. There was no effect of the presence or absence of eyes ($\chi^2 = 0.08$, df = 1, $P = 0.78$). We also found no effect of the original light condition from which the animals came ($\chi^2 = 2.32$, df = 1, $P = 0.13$). However, animals receiving the CWS with lights-on were markedly more responsive than those receiving CWS in darkness ($\chi^2 = 30.06$, df = 1, $P < 0.0001$). None of the two- or three-way interactions was significant and were therefore dropped from the model. These results are summarized in Figure 2.

Clearly, the light condition in which the animals receive CWS is a determining factor for their egg-laying response; this seems not to be influenced by the light condition in which they were kept prior to the CWS. Furthermore, the eyes seemed to play no role of importance in these effects.

Spontaneous egg laying

To determine whether *L. stagnalis* also had a preference to lay eggs during daylight hours without being stimulated by CWS, egg laying of 42 isolated animals was monitored for 96 h.

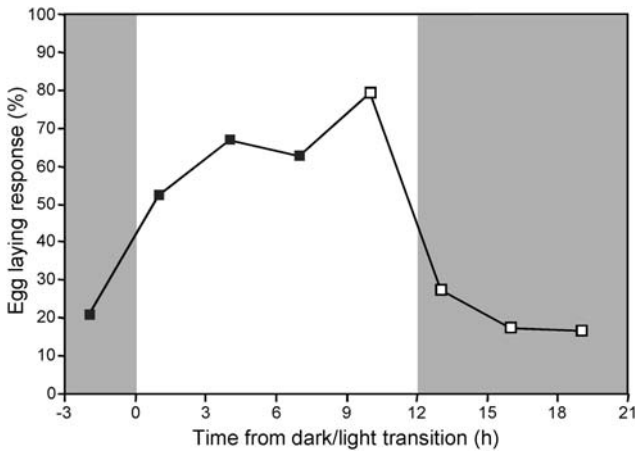

Figure 1. Diurnal changes in the effectiveness of the CWS in *Lymnaea stagnalis*. The percentage of snails laying an egg capsule within 3.5 h after stimulation is plotted against the time of day at the time of the CWS. The black data points are from the experimental series in which animals were kept under a reversed light/dark cycle.

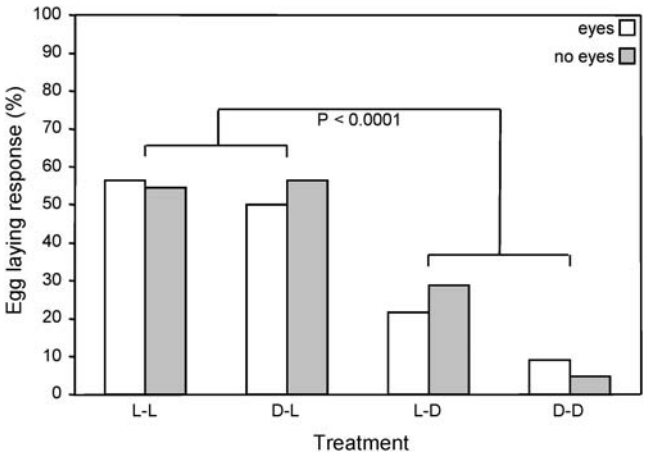

Figure 2. The role of the eyes and the presence of light in induced egg laying in *Lymnaea stagnalis*. For each treatment, L stands for light and D for dark. The first letter of each treatment indicates the light condition the animals were in prior to the CWS (original light condition), the second one indicates the light condition in which the CWS was given (CWS light condition).

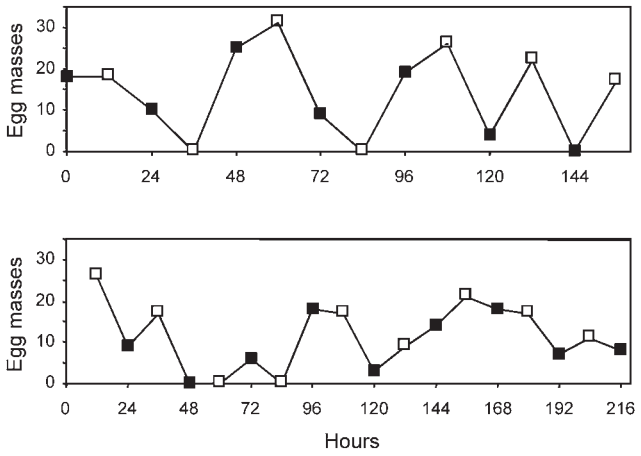

Figure 3. Egg-laying patterns of wild-caught *Lymnaea stagnalis* kept under natural light conditions. The graphs show egg laying during the night (black squares) and day (white squares) under natural light conditions. The top graph shows the results of the 52 animals caught in June, the bottom one those of the 57 snails caught in July.

Overall, during the night the animals produced a total of 21 egg masses, whereas they produced 38 during the day. The animals, therefore, tend to lay more eggs during the day ($\chi^2 = 4.32$, df = 1, $P = 0.038$).

The time elapsed since the animals' last egg-laying episode in animals laying eggs during the night was longer than that of animals that laid eggs during the day. Of the 14 night-time egg layers, 12 went without egg laying for more than 48 h. In contrast 12 out of the 18 animals that laid eggs during the day had laid eggs <48 h earlier (Fisher's exact test: $P = 0.0045$). In addition, the animals that laid eggs in the daytime showed a higher egg-mass output over the entire experiment than those that laid egg masses at night (3.1 ± 0.83 and 2.1 ± 0.61, respectively; Wilcoxon $\chi^2 = 9.85$, df = 1, $P = 0.0017$).

The pattern of egg laying in wild-caught snails is shown in Figure 3. In the first run (June) the 52 animals laid 85 masses during the night and 114 masses during the day ($\chi^2 = 4.24$, $P = 0.039$) in a 7-d period. In the second run (July) the 57 snails produced 83 masses during darkness and 118 masses during daylight ($\chi^2 = 6.13$, $P = 0.013$) in a 9-d period. As with the observations in the laboratory, there was a strong effect of day on whether eggs were predominantly laid during light or darkness (June: $\chi^2 = 57.33$, df = 6, $P < 0.0001$; July: $\chi^2 = 75.45$, df = 8, $P < 0.0001$).

DISCUSSION

Although it was already known that egg production varies seasonally in these animals (Wayne, 2001; Ter Maat *et al.*, 2007), the role of light perceived via ocular and/or nonocular photoreception in the regulation of this seasonality has not previously been investigated. Our data now reveal that the presence of light is important for the occurrence of CWS-induced egg laying in *Lymnaea stagnalis*. Besides finding a clear effect of light on CWS-induced egg laying, the data clearly show that the eyes are not necessary for this response to occur. The results were similar for animals with and without eyes, strongly suggesting that nonocular photoreceptors can mediate the response to light.

Most gastropod species have a pair of eyes for photoreception. However, light is not only perceived via the ocular system, but can also be detected via nonocular photoreceptors. For example, it has been shown for two species of the terrestrial slug genus *Limax* that the eyes are not necessary for the entrainment and maintenance of the circadian rhythmicity that these animals normally exhibit (Beiswanger, Sokolove & Prior, 1981). Also, in the sea hare *Aplysia californica* nonocular photoreception can modulate the circadian oscillator that governs long-term memory formation (Lyons, Rawashdeh & Eskin, 2006). Such nonocular photoreception is not exclusive to the Mollusca; for example, a recent study reported that scorpions (*Paruroctonus utahensis*) can still orientate themselves normally when their eyes are covered, by exploiting the light properties of their fluorescent cuticle (Gaffin *et al.*, 2012). In *L. stagnalis* studied here, the main nonocular photoreceptors are located in the skin (Van Duivenboden, 1982; Sunada *et al.*, 2010a, b). With the eyes, these snails can detect black and white check patterns (Andrew & Savage, 2000), while the dermal photoreceptors are, for example, responsible for the response to shadows (Stoll, 1973; Stoll *et al.*, 1976; Sunada *et al.*, 2010a, b).

The dermal photoreceptors seem to send input to the central nervous system via the inferior pedal nerves (Sudoplatov & Zhukov, 1999; Chono, Fujito & Ito, 2002), where the withdrawal response is mediated by input from these dermal photoreceptors into the interneuron Right Pedal Dorsal 11 (RPeD11; Sunada *et al.*, 2010a, b). This higher-order interneuron is located in the right pedal ganglion and is an important member of an electrically coupled network of neurons that mediate the escape withdrawal response in *L. stagnalis* (Syed & Winlow, 1989; Inoue *et al.*, 1996a, b). For example, its activity inhibits behaviours such as feeding, aerial respiration and locomotion, which are clearly incompatible with partial or full withdrawal into the shell (e.g. Lukowiak *et al.*, 1996; Hermann *et al.*, 1997). A remaining question is how this light information reaches the CDCs, which are ultimately responsible for the release of the egg-laying hormone CDCH. A second explanation could be based on the presence of light-sensitive carotenoids that are present in intracellular neuronal organelles, called lipochondria (e.g. Petrunyaka, 1982). Such carotenoids, as well as other photopigments, are thought to mediate the response to light of molluscan neurons (Baur *et al.*, 1977; Krauhs, Sordahl & Brown, 1977; Gotow & Nishi, 2009). *Lymnaea stagnalis* does have very brightly orange neurons, which might be due to the presence of such carotenoids (Petrunyaka, 1976). Although direct stimulation with light does not seem to evoke a response in the nerves emerging from the CNS (Stoll *et al.*, 1976), this does not exclude the possibility that subthreshold or inhibitory changes occur in these neurons (e.g. Brown & Brown, 1973; Kartelija, Nedeljkovic & Radenovic, 2003). Hence, further research is required to distinguish between the two possible explanations.

Clearly, light is only one of many factors that affect egg laying. For example, permanent grouping or frequent mating reduce egg output (Van Duivenboden *et al.*, 1985; Hoffer, Ellers & Koene, 2010), due to a seminal fluid component that is transferred upon insemination (Koene *et al.*, 2010). Also, animals more than 300-d old usually show a decline in egg production (Janse *et al.*, 1989; Janse, Ter Maat & Pieneman, 1990). In addition, low food availability (Bohlken *et al.*, 1986; Ter Maat *et al.*, 2007), infection by the schistosome parasite *Trichobilharzia* (de Jong-Brink *et al.*, 1992) and low temperatures (Vianey-liaud, 1981; Dogterom *et al.*, 1984) all inhibit egg laying.

It is reasonable to assume that reflexive egg laying in *L. stagnalis* has a threshold that varies according to environmental circumstances. This is exemplified by our result that more animals respond to the CWS by laying an egg mass when there is light. A possible mechanism may be provided by the finding of Antkowiak & Chase (2003) who demonstrated in

Helix aspersa that the electrical activity of the nerves projecting towards the ovotestis is strongly correlated with the number of ripe eggs. This kind of activity might well lower the threshold for environmental stimulation of egg laying in that species. This could also explain why, under the controlled conditions of a laboratory, *L. stagnalis* lays eggs on a regular basis in the absence of environmental stimulation and does not show a clear diurnal pattern (as reported here). Therefore, the presence of light serves as a permissive signal that facilitates egg-laying behaviour but interacts with other internal signals that are important for egg laying, such as the presence of ripe eggs and/or material for building the egg mass (Koene & Ter Maat, 2004; Ter Maat *et al*., 2007).

Similar simple neural analogues of motivation have been demonstrated in gastropods. In *L. stagnalis* the motivation to mate in the male role is determined by the seminal fluid content of the prostate gland. The size of the prostate is relayed to the central nervous system via a nerve (De Boer *et al*., 1997) and increases the motivation to copulate as a male.

In summary, we show that light does provide an important cue for egg laying in this species, which is in line with the decrease in oviposition activity found in *L. stagnalis* when kept in darkness (Van der Steen, 1967). These findings are clearly of importance for understanding overall activity patterns of molluscs (Lombardo *et al*., 2010; Stephenson & Lewis, 2011) and contribute to our understanding of the potential influence of light pollution on snail populations, especially in urban areas. The data on wild-caught snails are a first step towards understanding the adaptive value of the CWS and integrating this with knowledge about the underlying regulatory mechanism.

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