A NOTE ON OVIPOSITION BY *LYMNAEA STAGNALIS* (LINNAEUS, 1758) (GASTROPODA: PULMONATA: LYMNAEIDAE) ON SHELLS OF CONSPECIFICS UNDER LABORATORY CONDITIONS

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ABSTRACT: Oviposition by *Lymnaea stagnalis* (L.) on shells of conspecifics has been reported anecdotally from laboratory observations. In order to gain the first quantitative insight into this behaviour, we have quantified the proportion of individuals bearing egg clutches in a long-term monospecific outdoor laboratory culture of *L. stagnalis* during two consecutive late-summer months. The snails were assigned to size classes based on shell height. Differences between the size class composition of clutch-bearers and of the general population were statistically compared by means of Pearson's distance χ^2_P analysis. Egg clutches were laid on snails of shell height >15 mm (i.e. reproductive-age individuals), with significant selection for the larger size classes (shell height 25–40 mm). While the mechanisms of and reasons behind such behaviour remain unknown, selection of larger adults as egg-carriers may have ecological implications at the population level.

KEY WORDS: freshwater gastropods, *Lymnaea stagnalis*, great pond snail, reproductive behaviour

INTRODUCTION

Lymnaea stagnalis (Linnaeus, 1758) is a common Holarctic freshwater gastropod, inhabiting most of mainland Europe (BANK 2017), northern Africa, and large sections of Asia and North America. It is one of the best studied freshwater invertebrates and is often used as a model organism for laboratory studies on life history traits, behaviour, neurobiology, herbivory, ecotoxicology, etc. (e.g. NOLAND & CARRIKER 1946, TER MAAT et al. 1989, KOBAYASHI et al. 1998, BRIX et al. 2012). Both short- and long-term laboratory populations retain biological and ecological characteristics of their original wild populations (e.g. ELGER & BARRAT-SEGRETAIN 2002, NAKADERA et al. 2015), due to which the results can be extrapolated to field and natural situations. Virtually all such studies use laboratory populations of *L. stagnalis,* which can be easily raised for several generations in captivity on commercially available lettuce (e.g. NOLAND & CARRIKER 1946, TER MAAT et al. 1989, ELGER & LEMOINE 2005, GROSS & LOMBARDO in press).

The reproductive biology of *L. stagnalis* (which, like all other freshwater pulmonates, is hermaphroditic) is known in great detail at the molecular/cell and organismal levels (e.g. HERMANN et al. 1997, KOENE 2010, NAKADERA & KOENE 2013). However, its ecological aspects remain poorly known. In particular, oviposition on conspecifics' shells has remained only anecdotally described (NOLAND & CARRIKER 1946, DUSSART 1979), though *L. peregra* O. F. Müller 1774 (= *Radix balthica* Linnaeus, 1758 sensu SEDDON et al. 2014) appears to actively seek conspecifics' shells to lay their eggs, probably attracted by a possible calcium source for the hatchlings to come (PIGGOTT & DUSSART 1995). Some small-bodied planorbid and prosobranch species lay and carry around small egg clutches in the (functional)
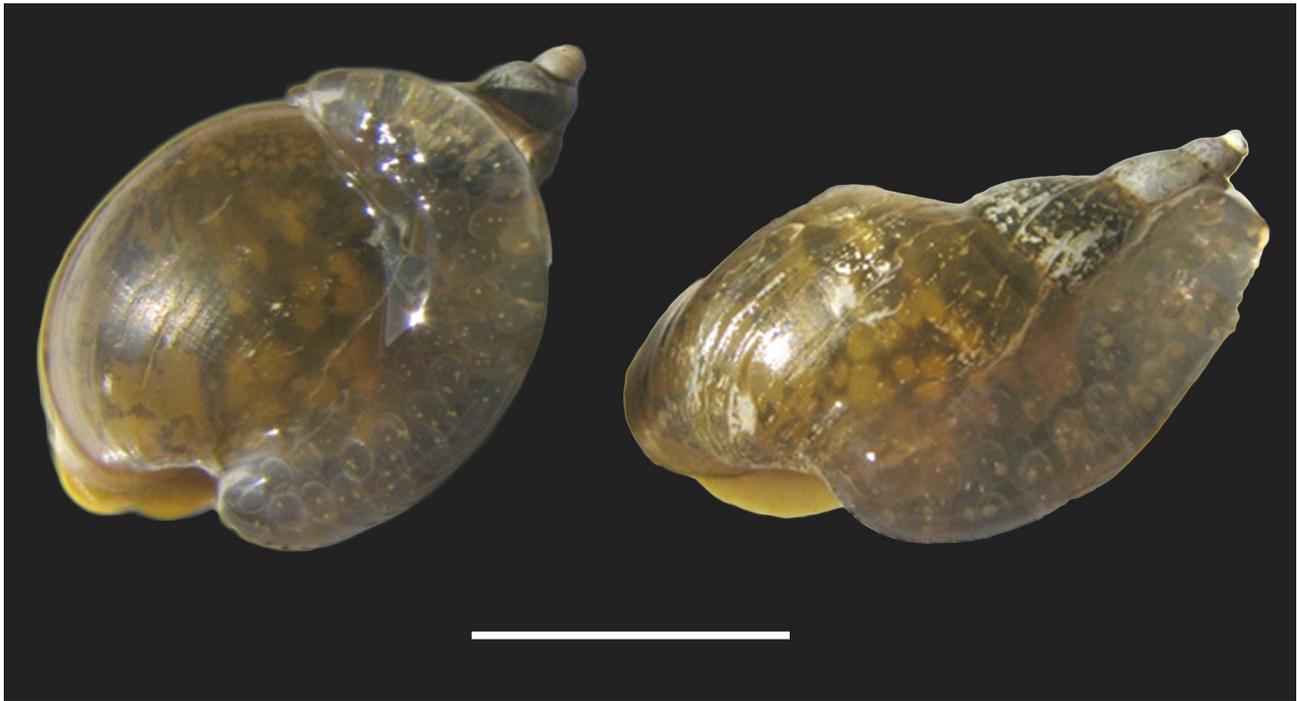
Fig. 1. Two medium-sized adults of *L. stagnalis* (shell height ~25–28 mm) from our culture with recently deposited egg
    clutches on their shells. Bar equal 1 cm

umbilical area of their own shells (ALBRECHT 2006, BOETERS & GLÖER 2007), while the larger-bodied lymnaeid *Radix auricularia* (Linnaeus, 1758) lay their clutches on conspecifics' shells, possibly as a response to large, irregular fluctuations in water level which could otherwise kill eggs attached to littoral substrata (ALBRECHT 2006). The large-bodied (adult shell height ~60 mm) *Benedictia pumyla* (Lindholm, 1924) has also been observed laying egg clutches on conspecifics' shells, probably as a result of the absence of other suitable hard substrata in their habitat (SITNIKOVA & SHIMARAEV 2001). Despite the highly calciphilous ecophysiology of *L. stagnalis* (PIGGOTT & DUSSART 1995, BRIX et al. 2012) and its occurrence on all kinds of substrata, there is almost no quanti-

tative information in the literature about its similar egg-laying behaviour.

We have repeatedly observed oviposition on conspecifics' shells in our long-term rearing aquarium of *L. stagnalis* (Fig. 1). In order to provide the first quantitative insight into this behaviour, we have quantified the number and size-class category of egg-carrying individuals of *L. stagnalis* in our outdoor aquarium during the summer peak of egg production. In particular, we wanted to ascertain if specific size classes of *L. stagnalis* were selected by ovipositing conspecifics (H_0: size class composition of clutch-bearing snails is the same as size class composition in the general population).

MATERIAL AND METHODS

The material included the entire monospecific, long-term culture of *L. stagnalis* in an outdoor laboratory in suburban Rome, Italy (41°43'N, 12°21'E). The aquarium was maintained in a patio area protected from direct sunlight, rain, strong winds, and the occasional near-freezing air temperature in winter. The snails were fed ad libitum with fresh lettuce occasionally mixed with fresh macrophytes and commercially available ornamental fish food in flakes to supply proteins and vitamins. A constant air flow (~90 L h⁻¹) was provided by a small pump for ornamental aquaria. The water was changed regularly, using calcium-rich, nutrient-poor tap water,

to avoid waste build-up which could have hindered snail growth and reproduction (e.g. LEVY et al. 1973). Continuous growth and reproduction at the individual and population levels indicated an adequate supply of calcium for the calcium-demanding *L. stagnalis* (BRIX et al. 2012). Our predator- and parasite-free *L. stagnalis* culture was started in 2009 from a few individuals originally collected in lower Lake Constance (Germany). For details of the culture conditions see LOMBARDO et al. (2011).

Observations were carried out in the second half of summer 2016 (July–September), when the egg production was at its annual peak. Observations
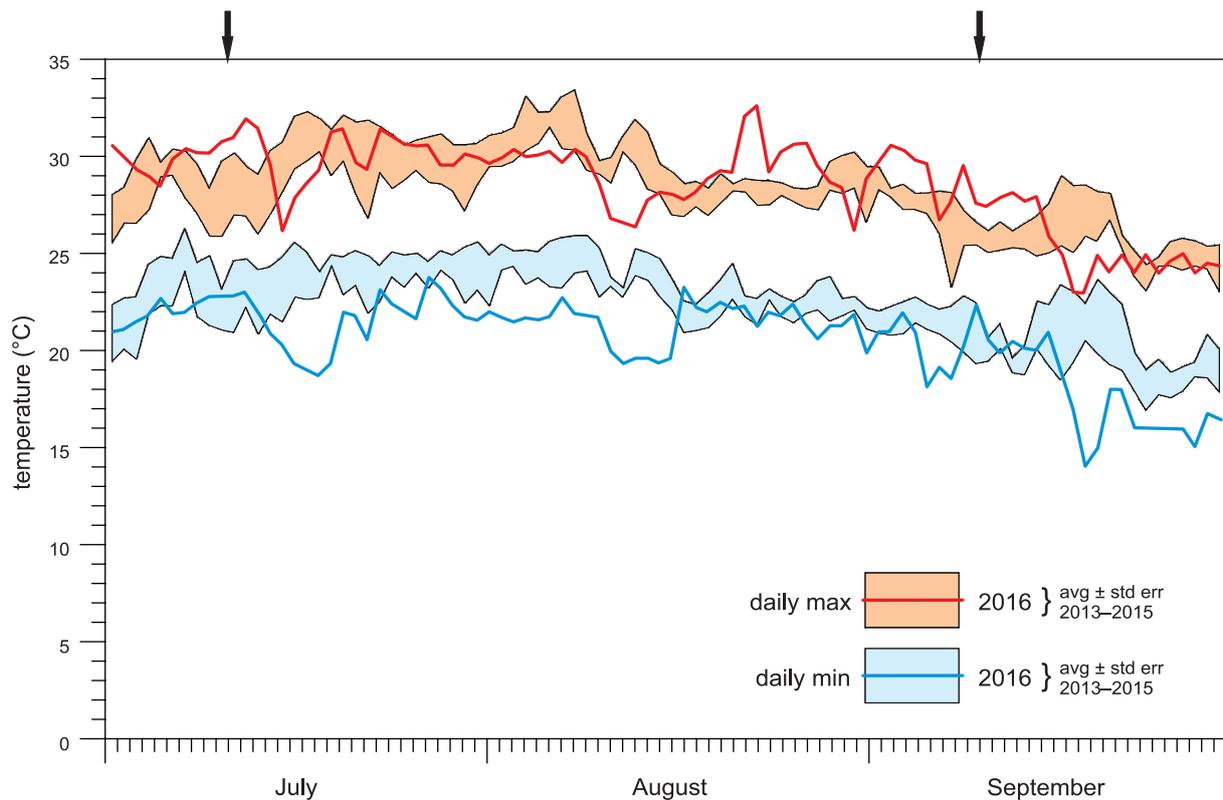
Fig. 2. Minimum and maximum daily air temperature at the aquarium in July–September 2016 (thick lines), against the average ± standard error from the previous three years (2013–2015; ribbons). Air and water temperatures were closely correlated (r = 0.996, n = 80, p << 0.0001) and were therefore treated as fully interchangeable. The arrows indicate the beginning (11 July) and end (10 September) of the clutch-bearing observations

on clutch-bearing snails encompassed two consecutive months, from 11th July till 10th September. Environmental and weather conditions remained stable and seasonable (Fig. 2), with a D : L < 12 : 12 photoperiod ranging from after summer solstice (11 July: D : L = 8 h 58 min : 15 h 02 min) to shortly before the autumn equinox (10 September: D : L = 11 h 14 min : 12 h 46 min). Egg-carrying individuals (Fig. 1) were identified during the weekly water changes and assigned to 5-mm shell height classes using an ad hoc size chart printed on laminated paper. Egg clutches were then removed and the snails were returned to their aquarium, thus leading to a with-replacement weekly sampling for clutch-bearing snails. Shifts to a larger size class by individual snails were assumed to have no effect on the choice by ovipositing conspecifics, as such shifts occurred for all size classes, so that individuals outgrowing their size class were replaced by smaller individuals in turn "promoted" to a larger size class, resulting in a relatively constant size-class distribution. Given the low numbers of weekly sampled clutch-bearing snails, the weekly counts of such snails were eventually pooled to obtain a single count number at the end of the July–September observation period.

The snail reproductive status (immature or adult) was associated with the size class on the basis of GROSS & LOMBARDO (in press), who found that the first oviposition occurred at ~21–22 mm shell height for lettuce-fed control snails from the population of *L. stagnalis* used in the present experiment. GROSS & LOMBARDO's (in press) and our *L. stagnalis* populations were kept under very similar temperature, food, predator- and parasite-free conditions.

A census of the entire culture of *L. stagnalis* was carried out at the end of the observation period (mid September). The snails were assigned to the same 5-mm size classes and counted. The eggs were excluded from the census. Such a census made up the "general population" of *L. stagnalis* against which the number of clutch-bearing snails was compared statistically to test whether any size class(es) in the general population was(were) selectively chosen by egg-laying individuals.

The statistical comparison was based on Pearson's χ^2 distance (χ^2_P) analysis of observed (clutch-bearing) vs. expected number of individuals (general population) after organising the data into a 2 (snail populations) × 8 (size classes) contingency table. Individuals in the two possible largest-bodied size classes – i.e., >40 mm in shell height – which are occasionally present in our culture, were not found during the observation period, and such size classes were excluded from the analysis, leaving a total of

eight size classes. Pre-reproductive individuals (shell height <15 mm) were not found to carry egg clutches, and a second, finer-scale analysis was carried out only on adults organised into a 2 (populations) × 5 (size classes) table.

Each dataset included one <5 datum, which may have led to an excessively liberal χ^2_P outcome (e.g. GOTELLI & ELLISON 2004, ZAR 2009). The more conservative Wilk's G^2-statistic (or likelihood ratio test) is deemed more appropriate for small samples (GOTELLI & ELLISON 2004, ZAR 2009), but a consensus is lacking about the best χ^2-based procedure for small samples (ZAR 2009: 473–474). Therefore, we ran parallel Wilk's G^2 tests as a way to check the reliability of the χ^2_P analysis. When a significant χ^2_P outcome was supported by a significant G^2 analysis, differences in relative distribution within individual size classes between the clutch-bearers and the general population were assumed for significant distances of the Pearson adjusted residuals associated with the χ^2_P analysis (XLSTAT 2014), as the G^2-based analysis does not allow for such pairwise comparisons. Because of the discrepancy between significant χ^2_P or G^2 outcomes and non-significant residuals results (probably derived from the presence of <5 data and/or from a higher conservatism of the residual analysis), we ×3-transformed the original data to remove the <5 observations and to obtain a meaningful analysis of adjusted residuals (e.g. GALASSI et al. 2014). Statistical analyses were run with Addinsoft™ XLSTAT®© v. 2014.6.04 with significance threshold pre-set at p = 0.05. Non-significant χ^2_P or G^2 outcomes (p > 0.05) were interpreted as concordance of size-class distributions in the clutch-bearing subpopulation and the general population, i.e. as absence of selectivity of clutch-receiving snails by egg-laying individuals.

RESULTS AND DISCUSSION

A total of 639 individuals comprised the general population in the aquarium, with the density of ~42 individuals L^{-1}. The low mortality and sustained reproduction suggest that the high-density rearing conditions were within the ecophysiological requirements of *L. stagnalis*, as long as the water was periodically changed and sufficient food provided. NOLAND & CARRIKER (1946) found a relatively high mortality and temporarily stunted growth only in an experiment with *L. stagnalis* density of ~79 individuals L^{-1}, also indicating that our snails were below severe overcrowding conditions.

The size-class distribution in the general population was uneven, but it was generally skewed toward young individuals, with a roughly bell-shaped distribution of reproductive-age adults, defined (for our laboratory population) as those with shell height ≥22 mm (GROSS & LOMBARDO in press) (Table 1, Figs 3 and 4). The uneven size class distribution was probably due to a temporary hiatus in hatching during the time that should have led to the mid-September 5–10-mm size class, as some eggs were removed for other experiments. A numerical bias toward young individuals is expected in *L. stagnalis*, a species with relatively short lifespan [~300–400 d in laboratory conditions: NOLAND & CARRIKER 1946, JANSE et al. 1990; but see e.g. BOAG & PEARLSTONE (1979)] for natural populations in low-food and/or low-temperature conditions] and with a high investment in reproduction (NOLAND & CARRIKER 1946, VAN DER STEEN et al. 1969).

Egg clutches laid on shells were found only on live snails. A total of 63 individuals, roughly corresponding to 10% of the general population, were found with an egg clutch attached to their shells (Fig. 1) in our two-month observation period (Table 1), though this might be an overestimate due to the with-replacement sampling. Pre-reproduction juveniles (shell height <15 mm) were never found with an egg clutch (Table 1, Figs 3 and 4). The size class distributions were significantly different across all size classes (Fig. 3) (Pearson's distance: χ^2_P = 127.990, Wilk's G^2 = 135.812; df = 7 and p <<0.0001 for both; all pairwise size class comparisons significantly different at p <0.05 as per analysis of Pearson adjusted residuals). When juveniles of <15 mm shell height were excluded from the analysis, the two bell-shaped size

Table 1. Frequency of occurrence (as number of individuals) of various size classes in the general population in the aquarium (sampled once at the end of the two-month observation period) and in the clutch-bearing population of *L. stagnalis* (as total counts from weekly samplings) in the July–September 2016 observation period; "–" = zero

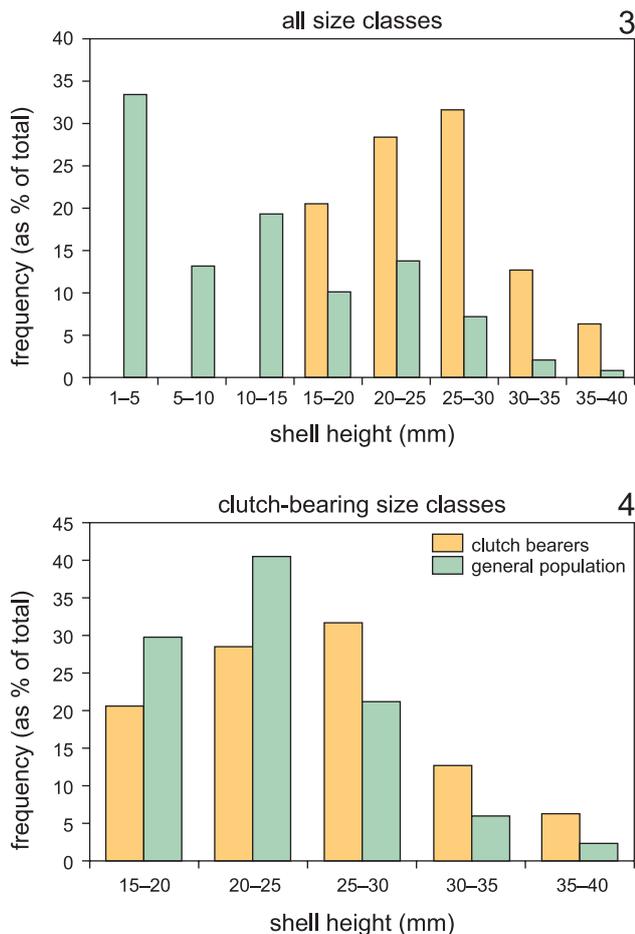| Size classes (shell height, mm)	Number of clutch-bearing individuals	Number of individuals in general population
1–5	–	214
5–10	–	84
10–15	–	124
15–20	13	65
20–25	18	88
25–30	20	46
30–35	8	13
35–40	4	5
40–45	–	–
45–50	–	–

Fig. 3–4. Percent distribution of 5-mm size classes (as shell height, in mm) within the general population in the aquarium (n = 639) and within the subpopulation of clutch-bearing *L. stagnalis* (n = 63) during (clutch-bearing snails) and at the end of (general population) the July–September 2016 observation period: 3 – all size classes; 4 – only the size classes comprising the clutch-bearing subpopulation are shown, with percent distribution of the same size classes in the general population recalculated for only such classes (i.e. excluding the juveniles: n = 217). All pairwise comparisons were statistically significant (p < 0.05) according to Pearson adjusted residuals analyses following significant χ^2_P and G^2 outcomes (details in text)

class distributions in the general population and in the subpopulation of clutch-bearers (Fig. 4) remained statistically different, albeit at a lower level of significance (Pearson's distance: χ^2_P = 11.093, p = 0.026; Wilk's G^2 = 10.454, p = 0.033; df = 4 for both), with frequency distributions statistically different (p < 0.05) within each size class according to the analysis of adjusted Pearson residuals for the 3×-transformed contingency table. The same residuals analysis of original data did not produce significant results despite the significant outcome of the associated χ^2_P analysis, probably due to different statistical power.

The absence of clutch-bearing juveniles (Figs 3 and 4) may be explained by the size of such snails

being simply too small to host even a small egg clutch. As in most freshwater pulmonates, the egg clutch size in *L. stagnalis* tends to follow the "female" body size (meant as the size of the parent snail in its female, or egg-producing, status) (e.g. GROSS & LOMBARDO in press: fig. 1b, c), though the clutch size may plateau or even taper off in older snails (NOLAND & CARRIKER 1946). The clutch size ranges in our population were <15–150 eggs or ~5–40 mm (LOMBARDO et al. 2011, GROSS & LOMBARDO in press, authors' personal observation), potentially rendering egg laying on small snails quite difficult if not impossible.

The egg-laying behaviour of *L. stagnalis* has been observed and described in detail by several authors (e.g. NOLAND & CARRIKER 1946, TER MAAT et al. 1989, HERMANN et al. 1997, KOENE 2010). Egg laying is triggered by neurological and hormonal signals that at first cause the soon-to-be ovipositing snail to (almost) stop locomotion and radular rasping, with the shell held in a slightly advanced and lowered position over the head, the tentacles curved downward, and the foot contracted (KOENE 2010: fig. 2c). Such a resting phase lasts 40–60 minutes (TER MAAT et al. 1989, KOENE 2010) and is followed by a ~60-min-long turning phase, during which locomotion is resumed, albeit within a small space, while the upraised and forward-oriented shell is repeatedly rotated sideways, and the surface is rasped ever more frequently. The direction of locomotion is also changed frequently. Such a turning phase is thought to prepare (clean) the surface where the eggs are about to be laid (TER MAAT et al. 1989, KOENE 2010), while the eggs travel inside the snail body to the female gonopore. Oviposition sensu stricto then follows, during which an egg clutch is deposited on and attached to the receiving surface while the parent snail glides slowly forward with sustained rasping. Oviposition proper lasts ~10–20 min, depending also on clutch size and speed of forward movement (e.g. KOENE 2010), "fast"-moving snails laying few eggs in much gelatinous matrix (NOLAND & CARRIKER 1946). The end of the clutch tapers off into a rounded point (authors' unpublished observations). A final inspection phase of ~10–30 min involves the snail moving slowly along the clutch brushing it with its oral lobes and tentacles; then the snail moves to the water surface to renew its air bubble (e.g. TER MAAT et al. 1989).

None of the detailed studies of egg-laying behaviour provides information as to whether soon-to-be-ovipositing snails actively seek a suitable space for their clutch, nor have we ever observed a snail displaying unusual behaviour or clearly directional locomotion before entering the egg-laying phase(s). In an elegant laboratory study by TER MAAT et al. (1989), experimental snails in which oviposition was hormonally and/or neurologically induced

either behaved as control snails or accelerated the egg laying ritual, skipping the initial resting phase and directly entering the turning phase, also suggesting that egg laying may be triggered by ovulation, which is in turn regulated by female hormones (KOENE 2010). Earlier observations in long-term *L. stagnalis* cultures under constant temperature, light, and photoperiod also suggest endogenous triggers of oviposition (VAN DER STEEN et al. 1969). All such patterns therefore suggest that oviposition occurs wherever the soon-to-be-ovipositing snail is when the eggs become ready for laying.

We did not directly observe oviposition, so it remains uncertain whether our *L. stagnalis* oviposited on their own shell or on conspecifics' shells. However, the position and direction of the oblong egg clutch on shells, with the clutch initially (stumpy end) deposited far from the female gonopore which is just inside the right side of the shell (Fig. 1), suggest that the clutches were laid by a donor snail different from the target snail. Oviposition on conspecifics' shells for the relatively large-bodied *L. stagnalis* is consistent with similar observations for other large-bodied species such as the lymnaeid *Radix* spp. (PIGGOTT & DUSSART 1995, ALBRECHT 2006) and the prosobranch *Benedictia pumyla*, a Lake Baikal endemic (SITNIKOVA & SHIMARAEV 2001), while small-bodied species may oviposit on their own shells (ALBRECHT 2006, BOETERS & GLÖER 2007). Allospecific oviposition on shells has been observed only for a small-bodied acroloxid limpet ovipositing on large shells of *Benedictia* spp. in Lake Baikal in the absence of suitable hard substratum (SHIROKAYA & RÖPSTORF 2003).

The absence of irregular growth patterns on snail shells and sustained growth and year-round reproduction (albeit much reduced in winter) suggest that the calcium requirements of the calcium-demanding *L. stagnalis* (PIGGOTT & DUSSART 1995, BRIX et al. 2012) were met in our aquaria by frequently refreshing the water. Therefore, it is unlikely that our *L. stagnalis* actively sought conspecifics' shells as a source of calcium for the future hatchlings, as suggested by PIGGOTT & DUSSART (1995) for *L. peregra* (= *R. balthica*) from soft-water habitats. The aquarium walls and bottom also provided plenty of hard substratum for egg laying, so that oviposition on shells by our *L. stagnalis* was not related to the unavailability of suitable substrata as observed for Lake Baikal gastropods (SITNIKOVA & SHIMARAEV 2001, SHIROKAYA & RÖPSTORF 2003). The water level in our aquarium was also stable, so that oviposition on shells by our *L. stagnalis* was unlikely to depend on water-level instability as ALBRECHT (2006) found for Lake Prespa endemic species. The egg-laying behaviour of *L. stagnalis* and the non-limiting calcium conditions suggest that oviposition in our aquarium was mostly random, though the relatively high-den-

sity conditions during our observations (~42 individuals L^{-1} and the aquarium walls covered with egg clutches) may have led some egg-laying snails to seek some "extra" space on the shells of nearby conspecifics. However, VAN DER STEEN et al. (1969: plate I-B, p. 136) report on laboratory-grown *L. stagnalis* laying eggs on conspecifics' shells at the much lower density of ~16 individuals L^{-1}. Oviposition activity in *L. stagnalis* is inversely proportional to density (MOOIJ-VOGELAAR et al. 1970), further complicating matters, and the influence of density on oviposition on shells remains unknown.

Larger adults (shell height 25–40 mm in Fig. 4 and related χ^2_P / G^2 analysis) were selected when eggs were laid on shells. We did not quantify the size of clutches laid on conspecifics, but most of the clutches were medium to full size (e.g. Fig. 1, LOMBARDO et al. 2011: figs. 1, A1), suggesting that most of the snails ovipositing on conspecifics' shells were full-grown adults (e.g. NOLAND & CARRIKER 1946 and authors' unpublished observations). Selection of larger adults is intuitive on the basis of the larger surface available for the pre-laying behaviour pattern described earlier (e.g. TER MAAT et al. 1989, KOENE 2010) and the relatively large space needed to receive most *L. stagnalis* clutches (e.g. Fig. 1, LOMBARDO et al. 2011: fig. 1). ALBRECHT (2006) found conspecific egg clutches on adult *Radix*, and SHIROKAYA & RÖPSTORF (2003) reported on allospecific egg laying on shells which involved a small-bodied species ovipositing on a large-bodied species, supporting our own observations on *L. stagnalis*.

We are unaware of similar observations of egg laying on conspecifics' shells in natural populations of *L. stagnalis*. The only other reports of egg laying on shells by *L. stagnalis* also come from the laboratory (NOLAND & CARRIKER 1946, VAN DER STEEN et al. 1969). Though *L. stagnalis* seems to retain its wild behaviour even after several laboratory-reared generations (ELGER & BARRAT-SEGRETAIN 2002, NAKADERA et al. 2015, GROSS & LOMBARDO in press), the ecological significance of oviposition on shells remains unknown. For example, lymnaeids and physids are among the fastest-moving freshwater gastropods (BOSS et al. 1984, HOFFMAN et al. 2006, KAPPES & HAASE 2012), and *L. stagnalis* in particular is capable of active horizontal dispersal (KOBAYASHI et al. 1998, KAPPES & HAASE 2012), suggesting that migrating clutch-bearing adults may increase the potential for colonisation of new habitats. *L. stagnalis* only oviposited on shells of live conspecifics, suggesting that such a behaviour may indeed have a strategic value for active colonisation of new habitats. However, whether the additional clutch weight decreases the colonisation potential by limiting the mobility of migrating clutch-bearing adults (especially on rough-surface or sandy substrata which slow down

horizontal crawling; Hoffman et al. 2006) remains unknown, as does the role of exogenous factors (e.g. parasitism) in oviposition on shells. We therefore urge further studies on the topic, especially at the population level and/or in natural conditions, to complement the current detailed knowledge of cell-, molecular-, and organismal-level of the reproductive biology and behaviour of *L stagnalis* (e.g. ter Maat et al. 1989, 2007, Janse et al. 1990, Hermann et al. 1997, Koene 2010, Nakadera et al. 2015).

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