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The effect of mating on female reproduction across hermaphroditic freshwater snails

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Abstract

Sexual conflicts often arise between mating partners because each sex tries to maximize its own reproductive success. One major male strategy to influence a partner's resource allocation is the transfer of accessory gland proteins. This has been shown to occur in simultaneous hermaphrodites as well as in organisms with separate sexes. Although accessory gland proteins affect the investment of resources in both male and female function, we here specifically focus on female investment. In the great pond snail, *Lymnaea stagnalis*, previous studies found that the accessory gland protein ovipostatin reduced female fecundity by suppressing egg laying in the partner in the short term (days). To investigate whether this reduction in egg laying is a commonly found effect of mating in freshwater snails, we compared egg output for evidence of suppression in isolated and paired snails of eight pulmonate species. Furthermore, we determined whether the suppression of egg laying caused a shift in resource allocation to the eggs. We found that in five of the eight species egg laying was suppressed, with fewer and lighter egg masses being laid when they had access to a mating partner. In mated pairs of *L. stagnalis* and *Biomphalaria alexandrina*, allocation of resources to the eggs was altered in opposite ways: individuals of *L. stagnalis* laid fewer but larger and heavier eggs; individuals of *B. alexandrina* laid smaller and lighter eggs, with no change in egg numbers. Such changes in the female function are most likely the result of combined effects of receiving accessory gland proteins, and the cost of mating in both male and female roles. Thus, effects of the maternal environment, including the receipt of accessory gland proteins, on offspring investment are not restricted to species with separate sexes.

KEYWORDS

egg, mollusc, reproduction, seminal fluid protein, sexual reproduction

1 | INTRODUCTION

During sexual interactions, reproductive optima often differ between the sexes as each sex tries to maximize its own reproductive success (Bonduriansky, 2001). Females generally invest relatively heavily in offspring production and, as a result, their reproductive success is largely determined by the choice of the right mating partner(s). Males invest substantially less towards offspring production, in terms of gametes; their reproductive success is therefore restricted by mate availability and thus mating opportunities (Bateman, 1948). What is more, traits that increase the reproductive success of one sex may be maladaptive to the other, thereby displacing one or both sexes from their evolutionary fitness optimum (Kazancıoğlu & Alonzo, 2012). Such interlocus conflicts can give rise to extreme and costly mating behaviors, setting off a sexually antagonistic arms race (Arnqvist & Rowe, 2005; Bonduriansky, 2001; Chapman, 2006). One typical example of sexual conflict is mating frequency, because males of many species have higher optimal rates of mating than do females (Chapman, Arnqvist, Bangham, & Rowe, 2003; Daly, 1978), while the costs of multiple mating in females are often relatively high. The cost of mating in females is not only due to the direct costs of mating, but also to physical damage that incurs survival and reproductive costs (Arnqvist & Nilsson, 2000; Kazancıoğlu & Alonzo, 2012; Rowe, Arnqvist, Sih, & Krupa, 1994; Wigby & Chapman, 2005).

Differences in reproductive strategies between the sexes find their origin in anisogamy (i.e., gamete size difference between the sexes) and should therefore also be present in hermaphroditic organisms (Schärer, Rowe, & Arnqvist, 2012). Recent research has indeed shown that sexual conflict does occur in simultaneous hermaphrodites and that it forms a driving force that is as important as that for the evolution of separate-sexed organisms. For example, sexual conflict is thought to have resulted in the evolution of seemingly harmful matings involving traumatic insemination in separate-sexed organisms such as bedbugs (Arnqvist & Rowe, 2005), strepsiptera insects (Peinert et al., 2016), and some cephalopods (Hoving et al., 2006), and in hermaphrodites such as sea slugs and polyclad flatworms (Anthes & Michiels, 2007; Michiels & Newman, 1998; Schmitt, Anthes, & Michiels, 2007); for a review see Reinhardt, Anthes, & Lange, 2015). Besides injection of sperm, hypodermic injection of accessory gland products alone can also be harmful, which is the case in land snails (Hasse, Marxen, Becker, Ehrenberg, & Epple, 2002; Koene, 2006; Schilthuizen, 2005) and earthworms (Koene, Pförtner, & Michiels, 2005; for a review see Zizzari, Smolders, & Koene, 2014).

Being both male and female, simultaneous hermaphrodites can strategically allocate shared reproductive resources to the sexual function with the highest reproductive gains (Koene, 2017). This reallocation often seems to be dependent on body size, because larger individuals lay substantially more eggs, allocating more towards their female function, while smaller individuals allocate proportionally more towards their male function (Hermann, Genereux, & Wildering, 2009; Mary, 1994; Petersen & Fischer, 1996; Schärer & Wedekind, 2001). In addition, simultaneous hermaphrodites can allocate resources to either their male or female function, depending on mating opportunity and partner quality (Charnov, 1979; reviewed in Schärer & Janicke, 2009). Studies on the great pond snail, *Lymnaea stagnalis*, found that sperm donors preferred new partners and that more sperm were transferred to virgins (Koene & ter Maat, 2007; Loose & Koene, 2008). Such flexibility, however, also opens up the possibility for manipulation, in which a sperm donor can alter the allocation of resources in a partner to benefit its own reproductive success (Hoffer, Schwegler, Ellers, & Koene, 2012; Michiels & Newman, 1998; Nakadera, Swart, Hoffer, et al., 2014).

One way of influencing a partner's allocation of resources is via accessory gland proteins (ACPs), often referred to as seminal fluid proteins when such proteins are transferred together with sperm in an ejaculate. Accessory gland proteins have been found to alter the behavior and/or physiology of the recipient in many species. In some insects, such as seed beetles, coccinellid beetles, fruit flies, and mosquitoes, these proteins induce post-mating responses such as a reduction in the willingness to re-mate (Lebreton et al., 2014; Perry et al., 2013). In *Drosophila melanogaster*, at least 138 identified accessory gland proteins seem to be transferred during mating (Findlay, Yi, MacCoss, & Swanson, 2008), and many of these proteins have been shown to each affect a specific aspect of the female physiology, such as increasing egg production (Avila, Ravi Ram, Bloch Qazi, & Wolfner, 2010; Hihara & Hirata, 1981; Ravi Ram & Wolfner, 2007, 2009; Rubinstein & Wolfner, 2013).

The role of seminal fluid components in simultaneous hermaphrodites has predominantly been studied in the pond snail *L. stagnalis* (but see Weber et al., 2018 for recent developments in *Macrostomum lignano*). These snails are capable of storing, digesting, and using sperm from multiple sperm donors, thus enhancing the level of sperm competition between rivals (Koene, Montagne-Wajer, Roelofs, & ter Maat, 2009; Nakadera, Swart, Hoffer, et al., 2014). Several studies indicate that high mating rates decrease egg laying, suggesting a probable effect of accessory gland proteins (de Visser, ter Maat, & Zonneveld, 1994; van Duivenboden, Pieneman, & ter Maat, 1985). In fact, ovipostatin (LyAcp10), an accessory gland protein produced in the prostate gland, was identified as the protein that suppressed egg laying by nearly 50% in the recipient (Koene et al., 2010), although the known physiological effects were short lived (less than a week). Studies have since shown that continuously-paired, as well as repeatedly-mated, individuals laid fewer eggs than individuals with less frequent mating opportunities, suggesting that higher mating frequencies displace females from their reproductive optima, even though the remaining weight per egg increases (Hoffer et al., 2012) and hatching success may increase (Hoffer, Mariën, Ellers, & Koene, 2017). As a result, it has been suggested that repeated receipt of ejaculates, caused by high mating rates, in combination with lower female fecundity indicate that sexual conflict also occurs in hermaphrodites that transfer accessory gland proteins via their ejaculate.

To investigate whether the effects observed in *L. stagnalis* are also present in other hermaphrodites, we compared snails within the molluscan clade Hygrophila, which are pulmonate freshwater snails belonging to the informal group Basommatophora (Jarne, David, Pointier, & Koene, 2010), to study allocation changes after recent

mating opportunities. Our eight study species, which included the model organisms *L. stagnalis* and *Biomphalaria glabrata*, comprised three different families (lymnaeids, planorbids, and physids). We tested whether mating opportunities would lead to a reduction in egg laying across snail species. If so, this would be an indicator that an ovipostatin-like effect evolved in the common ancestor of these three families. If, however, reduction in egg laying were unique to *L. stagnalis*, it would suggest that the evolution of this accessory gland protein is determined by more recent selection pressures. In addition, we quantified and analyzed morphological measurements of eggs, including dry weight, surface area, length, and volume, to determine whether egg laying suppression would result in a reallocation of resources to the individual eggs. Overall, our work deepens the knowledge from previous reports of changes in egg investment in *L. stagnalis* (Hoffer et al., 2012) and extends our understanding of egg investment in different species, showing a generality of reallocation in response to changes in egg laying.

2 | METHODS

2.1 | Study species

For this study we used eight simultaneously hermaphroditic species in the Hygrophila clade. The study species included five lymnaeids (*L. stagnalis*, *Pseudosuccinea columella*, *Stagnicola palustris*, *Stagnicola corvus*, and *Radix auricularia*), one physid (*Physa acuta*), and two planorbids (*B. glabrata* and *Biomphalaria alexandrina*). Our group at the Vrije Universiteit Amsterdam maintains several age-synchronized breeding tanks of the selected species in its molluscarium. Individuals of *L. stagnalis* (Linnaeus 1758) are easily reared under laboratory conditions, making this species a suitable model organism (Koene & ter Maat, 2005). The culture has been maintained for 50 years, originating from a wild population in a nature reserve and an agricultural area near Eemnes, The Netherlands. The culture of *P. acuta* (Draparnaud 1805) is a lab strain originating from the Netherlands that has successfully been reared for 20 years. The cultures of *S. palustris* (Müller 1774), *S. corvus* (Gmelin 1791), and *R. auricularia* (Linnaeus 1758) have been bred for nearly 5 years, and originate from Belgium and The Netherlands. As with *L. stagnalis*, all these species have a Holarctic distribution. *Pseudosuccinea columella* (Say 1817) is endemic to eastern North America but occurs over large parts of the Neotropics and has successfully been introduced into Europe. Our culture of this species originates from a greenhouse in Belgium and had been bred for nearly 5 years. *Biomphalaria glabrata* (Say 1818) came from a Brazilian lab strain 15 years ago and has a Neotropical distribution. *B. alexandrina* (Ehrenberg 1831) originates from the Nile region and has been bred successfully for 4 years.

The breeding and experimental laminar-flow basins for cultures of all species were maintained under similar environmental conditions at 20°C (±1°C), in oxygenated low-copper water, with a light:dark regime of 12:12 hr. The cultures were alternately fed broadleaf lettuce (*Lactuca sativa* L., frozen and then thawed for some species) or fish flakes (TetraPhyll, Tetra GmbH, Melle, Germany) three times per week (Koene & ter Maat, 2005).

All study species, except *P. columella*, generally mate unilaterally by performing a single sex role during a given mating, but can, after completing a unilateral sperm donation, reciprocate by switching roles (Koene & ter Maat, 2005; van Duivenboden & ter Maat, 1985). In *P. columella*, there is some evidence for a predominance of selfing over outcrossing (Gutiérrez, Yong, Wong, & Sánchez, 2002; Jabbour-Zahab et al., 1997; Nicot, Dubois, Debain, David, & Jarne, 2008). In addition, all the species lay gelatinous capsules filled with eggs, which we refer to as egg masses (Tompa, Verdonk, & van den Biggelaar, 1984). Within each egg is an embryo surrounded by nutrient-rich perivitelline fluid provided by the albumen gland (Plesch, Jong-Brink, & Boer, 1970). After having received sperm from a sperm donor, the recipient can store and use this sperm for the fertilization of its eggs several weeks to months after mating (Nakadera & Koene, 2013). All the adult individuals, when entering the experiment, had ample opportunity to donate and receive sperm.

2.2 | Experimental setup

In order to determine whether mating opportunities cause a reduction in egg laying, we compared the egg output of isolated snails to that of paired snails in the following experiment. For each of eight species, 65 age-synchronized adult snails were collected from our breeding facility (see Nakadera, Swart, Maas, et al., 2014 for details), where they would have had previous mating opportunities, and their shell lengths were measured before experimentation. The experiment consisted of two phases (see below): an 11-day pre-treatment phase, in which all snails were kept isolated; and an 11-day treatment phase, during which snails were either assigned to an isolated treatment (n = 20) or to a paired treatment (n = 40; Figure 1). In this way we could account for individual differences in fecundity by either comparing the same individual under isolated and paired conditions or by comparing egg laying of the same individual during two subsequent periods of isolation. Note that individuals of *L. stagnalis* can store sperm for up to 2 months (Nakadera, Blom, & Koene, 2014); thus, paired (single partner; Figure 1) and isolated (no partner; Figure 1) snails were assumed to lay outcrossed egg masses. Our focus was on the immediate effect of additional mating opportunities on female fecundity during the treatment phase, including the receipt of ACPs (ovipostatin in particular). Previous work by Hoffer et al. (2012) showed that in *L. stagnalis*, the presence of an inaccessible mating partner does not affect female reproductive investment. Based on those findings, we here assumed that investment changes were induced by actual mating, and for that reason (and to keep the experimental setup simple and manageable) we chose to contrast the isolated and paired treatment of adult snails only.

The first 4 days of the pre-treatment and treatment phase consisted of a standardization period, during which the individuals were allowed to accommodate to the new condition. For the pre-treatment phase, standardization meant isolating the snails, thereby

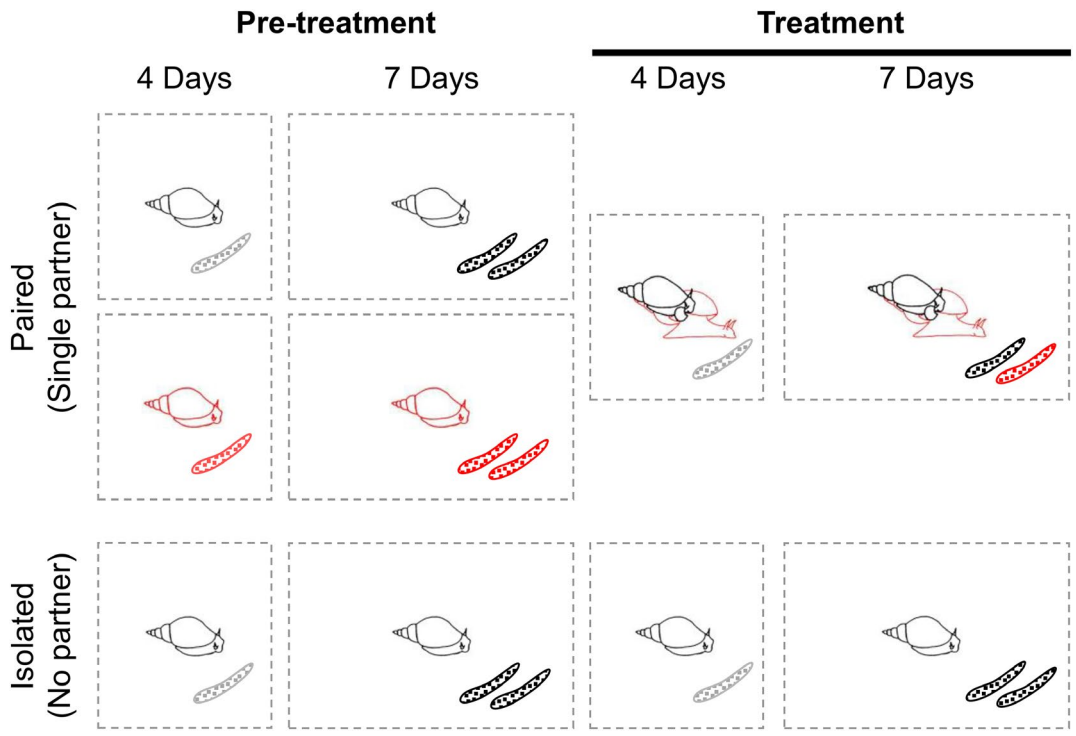

FIGURE 1 A schematic diagram of the experimental setup to test female reproductive output in several species of hermaphroditic freshwater snails. During the four-day acclimatization period preceding the pre-treatment and treatment weeks, all egg masses were discarded (acclimatization time, depicted as grey and pink egg masses). Egg masses were only collected during the two experimental weeks (seven days, depicted as black and red egg masses). The pre-treatment phase started once all snails were isolated, and the treatment phase started once the snails were divided into the two treatments, either remaining isolated (no partner) or being paired up with a size-matched partner (single partner, as indicated by black and red individuals). Note that there are no focal individuals within the pairs, and measurements from the egg masses produced by the paired black and red individuals were averaged. All snails were adults prior to the pre-treatment and are thus assumed to have sperm stored from previous mates that they use for producing outcrossed eggs

removing any direct physiological effects (including the recent receipt of ovipostatin) of previous copulations from their time in the lab culture (van Duivenboden et al., 1985), or to control for increases in egg laying due to clean-surface stimulus, which is triggered when snails are placed in clean containers (ter Maat, Lodder, & Wilbrink, 1983). Isolation also served to increase male motivation for mating (Koene, Loose, & Wolters, 2008; Swart et al., 2019). All non-laying individuals were removed from the experiment after the pre-treatment standardization period. For the treatment phase, standardization was needed to ensure copulation between the pairs occurred. Note that all egg masses laid during the standardization periods were discarded. Also, egg masses laid by individuals or pairs in which one of the partners died during the experiment were removed from the analysis.

Standardized snails remained isolated in perforated plastic containers (460 ml) for the pre-treatment week, during which time all egg masses laid were collected twice to determine each individuals' egg output during one week in isolation (Figure 1). In the treatment phase, all snails were ranked according to size and allocated to either an isolated treatment or a paired treatment, ensuring each treatment had a similar range in body size. The pairs were matched according to size (±1 mm), thereby removing size effect between partners (Nakadera, Swart, Maas, et al., 2014). As above, the standardization period was followed by a treatment week, during which time all egg masses laid by isolated and paired snails were collected twice to determine treatment effect on egg laying (Figure 1). Although not quantified systematically, pairs of all the species were seen mating during the experimental period, and most have been reported to mate multiple times within a few days after a period of isolation.

The number of egg masses, eggs per mass, as well as dry weight per egg mass and per egg were recorded for the two phases of the experiment. Egg masses were scanned at 150× magnification (Canoscan LiDE 700F Scanner), with a micrometer for scale, then freeze-dried (Lyph-Lock 6, Labconco, Kansas City, MO) to determine dry weight (microbalance, Mettler Toledo, UMT2, GmbH, Switzerland) per egg mass (g) and per egg. From the scans, the number of eggs per egg mass were later counted manually with the help of ImageJ Cell Counter plugin (http://rsb.info.nih.gov/ij/; http://rsbweb.nih.gov/ij/plugins/cell-counter.html). ImageJ was used to measure different variables (surface area and Feret's diameter: length and width) from the outer periphery of each of five randomly selected eggs from each egg mass. Using Feret's diameter, egg volume was calculated following the standard formula of an ellipsoid (for overview of methods see van Iersel, Swart, Nakadera, van Straalen, & Koene, 2014).

All statistics were performed with IBM SPSS 20. To reduce the effect of individual differences in reproduction, pre-treatment

week data were subtracted from the treatment week data per individual, and this difference in values was indicated with delta (δ). A Student's *t*-test was performed to determine the treatment effect on relative changes in fecundity (egg mass number, egg number, dry weight of egg mass) and reallocation of resources to eggs (egg dry weight, surface area, width, and volume). The number of eggs, egg masses, and total dry weight of egg masses were calculated per pair from the pre-treatment period by summing the egg output of both snails during isolation and dividing this by two. This was done so that the combined egg output during the treatment period could also be divided by two, to reflect output per snail. Individuals that did not lay egg masses during the treatment week were excluded from statistical analyses of variables associated with the reallocation of resources to eggs (see Table S1 for percentages of individuals from each species that did not lay eggs). The percent change between the paired and the isolated treatments was calculated for each egg variable during the treatment week as $[(\delta_{paired}-\delta_{isolated})/|\delta_{isolated}|] \times 100$, where "|" symbols indicate mean absolute value and δ is the calculated relative difference.

To address the phylogenetic relationships among the species used in this study, the maximum likelihood (ML) method was applied by using 16S mitochondrial gene sequences from GenBank (accession numbers: *B. alexandrina* AY030204.1; *B. glabrata* KF892020.1; *L. stagnalis* AY577461.1; *P. columella* U82073.2; *S. corvus* U82079.2; *S. palustris* U82082.2; *P. acuta* U038308.1; *R. auricularia* AF485646.1). To provide a robust rooting, we used *Latia neritoides* and an unidentified species of *Chilina* as outgroups (accession numbers: EF489307.1 and HQ659898.1, respectively). Sequences were aligned with MUSCLE and curated using Gblocks. The ML tree was built using PhyML and TreeDyn and tested for reliability by performing 1,000 bootstraps (Dereeper et al., 2008; Dereeper, Audic, Claverie, & Blanc, 2010). It should be noted that it was not our aim to reconstruct the full phylogeny of the freshwater snails, but to use this tree to assess the phylogenetic signal in the egg laying response. To do so, we used Blomberg's *K* with the phyloSignal function in the statistical packages Phytools and Ape within R software version 3.3.3 (Revell, 2012). The degree to which a trait shows phylogenetic signal predicted under Brownian evolution is indicated by *K* (*K* = 0 means that there is no phylogenetic signal, *K* < 1 means that closely related species weakly resemble each other, and *K* > 1 indicates that closely related species strongly resemble each other; Blomberg, Garland, & Ives, 2003). To obtain *p*-values of *K* we used 1,000 randomizations.

3 | RESULTS

3.1 | Relative change in female fecundity

In *L. stagnalis* we found a significant decrease in female fecundity of paired individuals compared to isolated snails, with a 70% reduction in the number of egg masses laid ($t(41) = 2.06$, $p = .046$), while a three-fold reduction was found in the number of eggs laid ($t(41) = 3.68$, $p = .001$; Table 1; see Table S1 for averages per species per treatment). In total, the paired snails laid lighter egg masses, decreasing total dry weight of egg masses by 140%, compared to isolated individuals ($t(41) = 2.9$, $p = .006$; Table S1).

Two of the other lymnaeids, namely *P. columella* and *S. corvus*, showed a similar decrease in fecundity. In both species, paired individuals laid significantly fewer egg masses than isolated individuals (*P. columella*, −1,065%, $t(31) = 3.13$, $p = .004$; *S. corvus*, −69%, $t(30) = 3.4$, $p = .002$; Table 1). Total egg numbers laid by pairs were significantly lower than for isolated individuals (*P. columella*, −887%, $t(31) = 2.83$, $p = .008$; *S. corvus*, −64%, $t(38) = 3.22$, $p = .003$). The total dry weight of egg masses decreased in the paired treatment (*P. columella*, −658%, $t(31) = 0.11$, $p = .03$; *S. corvus*, −51%, $t(38) = 2.77$, $p = .009$; Table S1). As in *L. stagnalis*, paired individuals of *P. columella* and *S. corvus* laid fewer and lighter egg masses, with a significant reduction in egg numbers compared to isolated snails (Table 1 and Table S1).

For the two planorbids, *B. alexandrina* and *B. glabrata*, the number of egg masses laid decreased as a result of pairing, though no

TABLE 1 The relative change in female fecundity (pre-treatment week subtracted from treatment week), for number of egg masses and eggs, between the paired treatment and the isolated treatment; $p < .05$ are indicated in bold. The percent difference and the corresponding difference δ (in parentheses) between the two treatments are also shown

		Relative number of egg masses				Relative number of eggs			
Family	Species	Isolated[a]	Paired[a]	*p*	% difference (δ)	Isolated[a]	Paired[a]	*p*	% difference (δ)
Lymnaeidae	*Stagnicola palustris*	−1.00 ± 1.04	−1.80 ± 1.13	.077	−36 (0.6)	−41 ± 22	−46 ± 52	.75	−5 (1)
	Stagnicola corvus	−3.25 ± 2.57	−5.50 ± 1.46	**.002**	−33 (1.7)	−49 ± 25	−80 ± 36	**.003**	−19 (8)
	Lymnaea stagnalis	−0.96 ± 1.25	−1.60 ± 0.81	**.046**	−36 (0.5)	−36 ± 89	−150 ± 113	**.001**	−54 (98)
	Pseudosuccinea columella	0.13 ± 1.45	−1.21 ± 0.95	**.004**	−33 (1.3)	−3 ± 38	−33 ± 21	**.008**	−42 (44)
	Radix auricularia	−1.90 ± 1.60	−2.50 ± 1.70	.26	−10 (0.5)	−64 ± 57	−69 ± 56	.81	−15 (23)
Physidae	*Physa acuta*	−2.60 ± 2.00	−3.50 ± 1.20	.22	−18 (1.1)	−110 ± 66	−122 ± 54	.64	−7 (10)
Planorbidae	*Biomphalaria glabrata*	1.35 ± 2.56	−1.93 ± 2.77	**.001**	−20 (3.3)	47 ± 155	24 ± 166	.66	27 (77)
	Biomphalaria alexandrina	4.04 ± 5.06	−5.20 ± 2.43	**.001**	−47 (8.5)	−47 ± 163	−29 ± 141	.70	−2 (10)

[a]Values are means ± *SD*.

TABLE 2 Change in allocation of resources to eggs: the relative change in egg dry weight and surface area (pre-treatment week subtracted from treatment week), between the paired treatment and the isolated treatment; $p < .05$ are indicated in bold

		Relative egg dry weight			Relative egg surface area		
Family	Species	Isolated[a]	Paired[a]	*p*	Isolated[a]	Paired[a]	*p*
Lymnaeidae	*Stagnicola palustris*	−3.09 ± 10.3	−5.45 ± 23	.80	−2 ± 30	−7 ± 43	.64
	Stagnicola corvus	−7.56 ± 10.1	−5.30 ± 4.4	.37	−13 ± 45	−3 ± 22	.38
	Lymnaea stagnalis	−11.6 ± 5.4	−0.41 ± 16	**.005**	−68 ± 65	108 ± 65	**.001**
	Pseudosuccinea columella	−0.80 ± 4.7	0.20 ± 3.5	.49	−14 ± 41	3 ± 41	.23
	Radix auricularia	−2.65 ± 16.1	−0.73 ± 8.8	.64	−24 ± 58	−21 ± 2	.82
Physidae	*Physa acuta*	−2.75 ± 7.7	−5.60 ± 3.4	.26	−43 ± 80	−59 ± 51	.58
Planorbidae	*Biomphalaria glabrata*	8.50 ± 17.9	2.90 ± 13	.26	143 ± 204	31 ± 186	.077
	Biomphalaria alexandrina	3.30 ± 6.7	−4.90 ± 7.9	**.001**	156 ± 77	9 ± 69	**.001**

[a]Values are means ± *SD*.

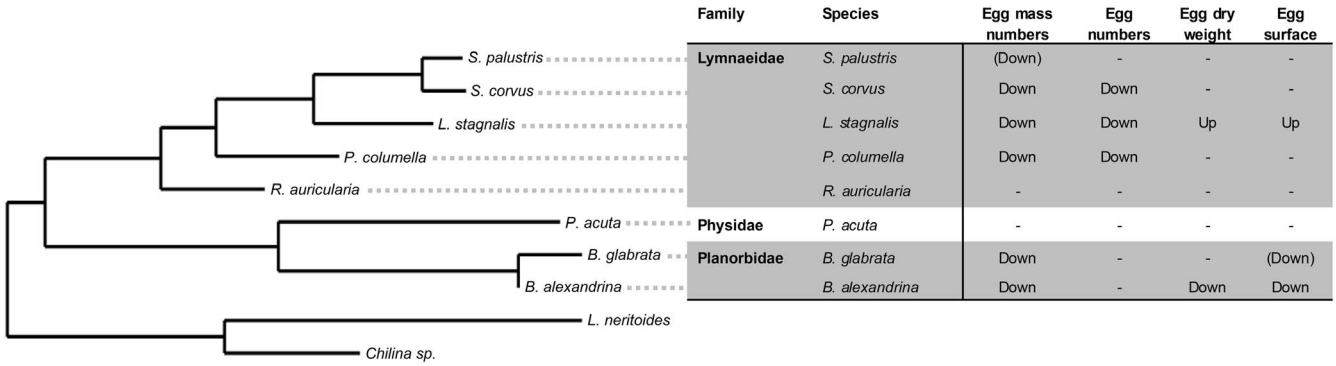

FIGURE 2 Summary of the results and phylogeny of the species used in this study of female reproduction in hermaphroditic freshwater snails. The maximum likelihood phylogenetic tree is shown on the left side, with *Latia neritoides* and a species of *Chilina* as outgroups. On the right side, a summary table of the results for the different egg laying parameters is shown (each row corresponds to each species used in this study). Significant differences between paired and isolated treatments are indicated by the direction of change of each variable ("Up" or "Down"). Parentheses indicate a nonsignificant trend

difference was found in the number of eggs laid. A greater than two-fold decrease was found in the number of egg masses laid by paired individuals of *B. alexandrina* and *B. glabrata* compared to isolated counterparts (*B. alexandrina*, −229%, $t(41) = 7.45$, $p = .001$; *B. glabrata*, −243%, $t(38) = 3.88$, $p = .001$; Table 1). No difference in the number of eggs laid by either species was found between the two treatments (*B. alexandrina*, $t(41) = -0.38$, $p = .7$; *B. glabrata*, $t(38) = 0.438$, $p = .66$). Total dry weight of egg masses, however, did decrease significantly in the paired treatment in both the species (*B. alexandrina*, −91%, $t(41) = 2.27$, $p = .03$; *B. glabrata*, −376%, $t(38) = 4.76$, $p = .001$; see Table S1). Thus, paired individuals of both species of *Biomphalaria* laid fewer and lighter egg masses, but no change in egg numbers was detected compared to isolated snails, although this was, in part, due to high variation (Table 1 and Table S1).

No change in female fecundity was found in the remaining lymnaeids, *S. palustris* and *R. auricularia*, nor in *P. acuta*, with egg mass number (Table 1), egg number, and dry weight of egg masses not significantly different between isolated and paired snails ($p > .05$; Table S1). However, there was a trend for paired individuals of *S. palustris* to lay fewer egg masses than isolated individuals (−81%, $t(23) = 1.85$, $p = .077$).

3.2 | Change in allocation of resources to eggs

Paired individuals of *L. stagnalis* laid heavier and larger eggs than isolated snails. In *L. stagnalis*, the change in the dry weight of eggs laid by paired snails did not differ between the 2 weeks, while a significant decrease in egg weight was detected in isolated snails (96%, $t(36) = -3.02$, $p = .005$). Relative egg surface area, egg width, and egg volume all increased in the paired treatment but decreased in the isolation treatment (for all species, $p < .01$). Egg surface area was thus used as a proxy for egg size as it adequately represented the other egg measurements (Table 2 and Table S1). A 271% increase in egg size was found after pairing compared to isolation ($t(36) = -8.14$, $p = .001$; Figure 2; Table 2 and Table S1).

In *B. alexandrina*, the reduction in number of egg masses in the paired treatment was not associated with a reallocation of resources to the eggs; in isolated individuals, egg weight and size increased

over time, whereas paired snails laid significantly lighter and smaller eggs (egg weight, −251%, $t(41) = 3.69$, $p = .001$; egg size, −94%, $t(41) = 6.53$, $p = .001$) than their isolated counterparts (Figure 2). In *B. glabrata*, there was no difference in relative egg dry weight between the treatments, although there was a tendency for paired snails to lay smaller eggs (−78%, $t(38) = 1.82$, $p = .08$; Figure 2; Table 2 and Table S1). None of the other species showed a significant reallocation of resources to the eggs (Table 2 and Table S1).

3.3 | Phylogeny

In order to assess the phylogenetic relationships among the species tested, we performed a phylogenetic reconstruction analysis which resulted in a tree (Figure 2) that is consistent with the phylogeny of Basommatophora (e.g. Correa et al., 2010; Dayrat et al., 2011). We found no significant association between presence/absence of a change in egg laying and phylogenetic position of the test species, but we did find that closely related species resembled each other in their quantitative response (egg masses, $K = 0.975$, $p = .023$; egg numbers, $K = 1.081$, $p = .008$), which is consistent with our findings that pairing affected egg number only in the Lymnaeidae, and that the directions of change in egg dry weight and surface area were opposite in the Planorbidae compared to the Lymnaeidae.

4 | DISCUSSION

Our findings highlight that the effects of the maternal environment, including the presence of a mate, on offspring investment and possibly fitness are not restricted to species with separate sexes. For five of the eight hermaphroditic snail species we investigated, female reproductive investment was altered by having the opportunity to mate. When comparing the egg production of paired snails to isolated snails, we found that egg laying was suppressed after pairing in the majority of species that we tested. However, this reduction in egg production was not associated with an increased investment of resources per egg in any of the species except in *L. stagnalis*. In fact, in both species of *Biomphalaria*, individuals in the paired treatment showed a reduction not only in the number of egg masses laid but also in the investment per offspring (measured as either weight and/or size of eggs), leading to an overall decrease in reproductive investment when mated. In summary, these results provide evidence for a common phylogenetic signal that is indicative of an ovipostatin-like effect in multiple species, but also suggests that other factors may be involved in determining egg size. The possibility that ovipostatin may be involved is supported by the recent report that sequences matching the gene that encodes this accessory gland protein were found in the genome of *B. glabrata* (Adema et al., 2017).

The reduction in egg laying found in the paired treatment could be due to high energetic investment in mating in either the male or the female role, causing a reallocation of resources away from egg laying in the short term. A high cost of mating in either sex role has been indicated by numerous studies on insects (Arnqvist & Nilsson, 2000), ungulates (McElligott, Naulty, Clarke, & Hayden, 2003), pinnipeds (Deutsch, Haley, & Le Boeuf, 1990; Galimberti, Sanvito, Braschi, & Boitani, 2007), and primates (Thompson & Georgiev, 2014), to name a few. This would reflect a direct trade-off in the allocation of resources between the sexes predicted by sex allocation theory (Charnov, 1979; Schärer & Janicke, 2009). Isolated snails that are previously mated and non-selfing are restricted to the female role, and they therefore only lay eggs and do not receive or donate ejaculates. By contrast, paired snails must divide their resources between both sex roles (egg laying, courtship behaviors, ejaculate production). Hoffer, Ellers, and Koene (2010) found that individuals of *L. stagnalis* that were experimentally restricted solely to the male function (sperm donation) experienced a similar reduction in fecundity as those mating reciprocally (in both sex roles; receiving and donating ejaculates). The high costs of mating in the male role (courtship behaviors, producing and delivering an ejaculate containing sperm and accessory gland proteins) can cause a direct reallocation of resources from female to male function within the same individual (Hoffer et al., 2010). Similar suppression in egg laying after mating or after grouping (in studies where copulations were not monitored) seems to be quite widespread across hermaphroditic gastropods; it has been reported in the sea hare *Aplysia brasiliana* (Blankenship, Rock, Robbins, Livingston, & Lehman, 1983), the land snail *Bradybaena pellucida* (Kimura & Chiba, 2015), and the freshwater snail species *Lymnaea elodes* (Florin et al., 2000), *B. glabrata* (Thomas & Benjamin, 1974), *Bulinus truncatus* (Bayomy & Joosse, 1987), and *P. columella* (Gutiérrez et al., 2002). Although the experimental setups and research goals were different in those studies, the similarity among the effects is evident.

Reduced fecundity observed in the paired snails may also be due to the direct costs of mating in the female role (Daly, 1978; Rowe, 1994), the receipt of excess sperm (Nilakhe, 1977), or the receipt of ejaculates containing accessory gland proteins that are harmful to the female (Eberhard & Cordero, 1995; Fowler & Partridge, 1989; Gems & Riddle, 1996). Hoffer et al. (2010) found that individuals of *L. stagnalis* that were experimentally restricted to the female function (receiving ejaculates) showed a similar reduction in egg laying to those allowed to mate reciprocally, suggesting that female mating costs for this species may be high due to the receipt of both excess sperm and accessory gland proteins such as ovipostatin, which is known to suppress egg laying (Hoffer et al., 2010; Koene, Montagne-Wajer, & ter Maat, 2006).

Some species, namely *P. acuta*, *R. auricularia*, and *S. palustris*, showed no change in fecundity after mating. This could be due to a number of different factors: individuals of these species did not copulate at all or only infrequently; the costs of mating are not high enough to elicit a change in fecundity; females have adapted to counter the effect(s) of accessory gland proteins that are transferred in an ejaculate; and/or males of these species do not invest in costly

accessory gland proteins to affect the partner's physiology (note that these factors are not mutually exclusive). As a result, individuals of these species may not show resource allocation away from the female function.

Any of the mating costs associated with the male or female role reduce the resources available for the female function, which may affect the rate at which the female organs involved in egg production recover after egg laying. For example, the albumen gland, which provides perivitelline fluid containing carbohydrates and proteins to the eggs, has been shown to restore almost completely in the time it takes to form a new egg mass (±32 hr) (Nagle, Akalal, & Painter, 1999; Wijsman & van Wijck-Batenburg, 1987). As already shown by Koene and ter Maat (2004), snails that lay many eggs deplete their albumen gland; the degree to which this gland is full may provide a signal for egg laying. In addition, starvation (low resources) affects albumen gland enzyme activity and substantially reduces egg laying (Wijsman, 1989). Thus, a delay in repletion of the albumen gland could affect egg laying rates by limiting the number of eggs that can be provisioned and/or could result in smaller eggs.

Given this framework, one could hypothesize that females with lower albumen gland recovery rates have three main strategies in the trade-off between offspring numbers and size (quantity–quality), in which finite resources need to be partitioned between these different components of fecundity (Smith & Fretwell, 1974; Winkler & Wallin, 1987). The first is to delay egg laying, with an overall decrease in egg numbers and no reallocation of resources to egg provisioning, as we found in *P. columella* and *S. corvus*. The second is to delay egg laying, with no change in egg numbers but instead lay smaller eggs, as we found in *B. glabrata* and *B. alexandrina*. And third is to delay egg laying, and to lay fewer eggs, but maintain or increase the provisioning per egg, as in *L. stagnalis*. In accordance with Hoffer et al. (2012), for the latter species, we found that egg production decreased as a result of mating, but egg size increased. The eggs that were laid were not only heavier but also larger, suggesting an increase in albumen gland products towards egg provisioning rather than a thicker gelatinous matrix surrounding the eggs. Being larger or better provisioned may affect hatchling survival and success (Moran & McAlister, 2009), although the adaptive advantage may depend on the environment (Hoffer et al., 2017).

Which of the three allocation strategies outlined above is most beneficial for each species depends on environmental factors, among others, which affect the relationship between egg size and offspring fitness. Egg size and mass shape have been shown to be important to meet the oxygen demands of the embryo, and can affect developmental temperature of the eggs (Duellman & Trueb, 1986). For example, in the frog species *Rana temporaria*, it was found that not only did larger eggs retain heat better than smaller eggs, but that globular egg masses dissipated heat better than disc-like masses (Duellman & Trueb, 1986; Wells, 2010). In *L. stagnalis*, a species with a Holarctic distribution, the larger eggs laid by mated snails may retain heat better and therefore develop faster and hatch earlier. Given the reduced resources available for mated snails, it may be that *L. stagnalis* copes best by increasing egg size. Additionally, the globular egg masses of *L. stagnalis* may retain heat better, potentially being warmer than the surrounding water, while the gelatinous layer surrounding the eggs is porous enough for the dissolved oxygen to reach the centrally located eggs. However, a contrasting pattern was found in mated pairs of *B. alexandrina* and *B. glabrata*, in which egg numbers did not change, but egg size decreased, suggesting a different evolutionary strategy (Smith & Fretwell, 1974; Winkler & Wallin, 1987). The habitat of these species is rather different. *Biomphalaria glabrata* is Neotropical and *B. alexandrina* comes from Egypt. In such warmer waters, the eggs of the mated snails in this genus may not suffer too much from being smaller, as the lower oxygen demands of small eggs minimizes hypoxia (Moran & Woods, 2007). Chronic hypoxia in a salamander species was found to increase developmental time, delay hatching, and produce hatchlings that were less developed (Mills & Barnhart, 1999). In addition, the disc-like egg masses of the two species of *Biomphalaria* (Hathaway, Adema, Stout, Mobarak, & Loker, 2010) may dissipate heat better and potentially be lower in temperature than the surrounding water, while exposing each egg to the water for oxygen, often a limiting factor in warm water. Both egg size and mass shape have been shown to be adaptive to the oxygen demands of the embryo (Duellman & Trueb, 1986). As such, previous findings support that the trade-off between offspring number and size and egg mass shape is governed in part by environmental factors, with females opting for the strategy that will maximize their reproductive success.

A trade-off between offspring number and offspring quality in response to the maternal environment is consistent with life history that maximizes maternal fitness (Leips, Richardson, Rodd, & Travis, 2009; Smith & Fretwell, 1974). In *L. stagnalis*, individuals can adjust offspring quality to seasonal changes by providing more serotonin to eggs earlier in the year or when population densities are high, thereby increasing developmental and behavioral characteristics such as enhanced locomotion for better dispersal potential (Ivashkin et al., 2015). This supports work on separate-sexed organisms, in which the maternal environment (population density and environmental stress) influences investment to the offspring, such as in *Orchesella cincta* (Liefting, Weerenbeck, Van Dooremalen, & Ellers, 2010; Zizzari, Braakhuis, van Straalen, & Ellers, 2009), least killifish (Leips et al., 2009), moor frogs (Räsänen, Laurila, & Merilä, 2005), apple snails (Ichinose & Tochihara, 2003), black widow spiders (Johnson, Miles, Trubl, & Hagenmaier, 2014), and dusky sharks (Hussey et al., 2010).

For future research, simple experiments could determine the validity of the three allocation strategies outlined above, and test whether there is an effect on albumen gland recovery rates after insemination and whether offspring from isolated and paired snails differ in survival and fitness at different temperatures. Assessing the average dry weight of the gland before egg laying (from isolated snails), then, through a time series and monitoring albumen gland recovery rates of isolated and paired snails would indicate whether the albumen gland recovers slower in the mated pairs. Obtaining eggs from isolated and paired snails and comparing their survival rates at varying temperatures would indicate whether larger eggs of paired *L. stagnalis*, being better provisioned, would

survive better at lower temperatures, and whether smaller eggs laid by paired individuals of *Biomphalaria* spp., do nearly as well as the larger eggs of isolated snails at warmer temperatures. Both experiments would aid in elucidating the mechanisms behind the quality–quantity trade-off.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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