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Evaluating the contributions of change in investment and change in efficiency to age-related declines in male and female reproduction

J. R. AULD*, P. JARNE†, V. SARDA†, H. JOURDAN-PINEAU†[1], T. LAMY†, B. PÉLISSIÉ† & P. DAVID†

*Department of Biology, West Chester University, West Chester, PA, USA
†Centre d'Ecologie Fonctionnelle et Evolutive – UMR 5175, Campus CNRS, Montpellier Cedex, France

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sex allocation.

Abstract

It is commonly observed that reproduction decreases with age, often at a different rate in males and females. This phenomenon is generally interpreted as senescence. Such reproductive declines may stem from at least two sources: a change in resource allocation and a decline in the ability to convert resources into offspring. This distinction is important because a shift in resource allocation may be favoured by selection, while reduced efficiency is purely deleterious. We propose a way to distinguish whether a decline in reproduction is purely deleterious based on estimating reproductive investment, output, and their ratio, efficiency. We apply this approach to the hermaphroditic snail *Physa acuta* and demonstrate that both male and female functions decline with age. The male decline largely stems from reduced investment into male activity while female decline is due to increased reproductive inefficiency. This shows that age-related declines in reproduction can occur for a number of different reasons, a distinction that is usually masked by the general term 'senescence'. This approach could be applied to any species to evaluate age-related reproductive decline. We advocate that future studies measure age trajectories of reproductive investment and output to explore the potential processes hidden behind the observation that reproduction declines with age.

Introduction

Senescence, an intra-individual deterioration in fitness with age, is widely documented in both short- and long-lived species under laboratory and field conditions (e.g. Rose, 1991; Charmantier *et al.*, 2006; Escobar *et al.*, 2008). Senescence is a deleterious process that can reduce fitness by shortening lifespan and decreasing reproductive output; it results from the expression of mutations that are deleterious to late-expressed traits (Williams, 1957; Rose, 1991; Kirkwood, 2002). Often, any decline in fitness with age is attributed to senescence, a broad view of the phenomenon that encompasses all manner of explanations for why, for example, the number of offspring produced may decline with age (Rose, 1991; Promislow, 2003). This diversity of mechanisms has led some authors to make the distinction between aging (a change in a trait with age) and senescence (a purely deleterious change with age) (e.g. Jones *et al.*, 2014). Whether or not one accepts these definitions, it is important to envisage the fact that not all changes with age are necessarily interpretable as purely deleterious, even if these changes are declines in reproductive output. A narrower focus on the mechanisms of decline, which distinguishes patterns of resource allocation, may shed light on this issue. For example, if an individual decreases allocation to a given function as it ages, relatively less will be gained through that function with age, but not necessarily because the function itself is impaired. A function can decline with age because less energy is invested into it (i.e. a change

Correspondence: Josh R. Auld, Department of Biology, West Chester University, 750 S. Church St., West Chester, PA 19383, USA.
Tel.: 610 436 0046; fax: 610 436 2183; e-mail: jauld@wcupa.edu
[1]Present address: CIRAD, UMR CBGP, Montpellier, France.

in allocation), because each unit of energy spent for that function produces less fitness gains (i.e. a change in efficiency), or both. When different functions are expressed in the same individual, energy not allocated to one function might be redirected to the other (e.g. De Visser *et al.*, 1994), whereas energy invested into an inefficient function is lost. Reallocation might occur, for example, between reproduction and helping kin or between the male and female functions in hermaphrodites. More generally, functions that utilize resources differently are likely to show different patterns of age-related decline.

One obvious example of differential resource utilization is seen by comparing males and females. Many studies have reported sex-specific differences in longevity where females outlive males (e.g. Fox *et al.*, 2003; Catchpole *et al.*, 2004). This observation has been linked to the idea that female reproduction is typically limited by resource availability, whereas male reproduction is limited by competition for mates. Thus, selection on males may be stronger at any particular time, especially early in life, and senescence in males may be faster than in females (Williams, 1957; Promislow, 2003). This idea has been extended to explain sex-specific senescence of reproductive traits (Reid *et al.*, 2003; Bonduriansky *et al.*, 2008; Nussey *et al.*, 2009). However, patterns of sex-specific declines in reproductive output with age can arise either from a decrease in the quantity of resources used for reproduction (i.e. a decline in allocation) or from a decline in the ability to utilize resources effectively (i.e. to efficiently convert resources into viable offspring). The distinction is important because patterns of resource allocation can be optimized by selection, whereas a decline in the ability to efficiently utilize resources is purely deleterious (although it may represent a by-product of positive selection on early-expressed traits under the antagonistic pleiotropy theory).

To date, most studies on senescence have focused on species with separate sexes where resources can be reallocated from reproduction to growth (and therefore future reproduction), to survival or to helping kin (Bell, 1980; West *et al.*, 2002; West, 2009). Thereby, changes in reproductive output may reflect adaptive strategies to maximize total lifetime (inclusive) fitness. Even in species where kin selection is weak, a decline in reproductive investment with age should not necessarily be interpreted as the result of an age-related deterioration in the ability to utilize resources. Rather, it may be that allocation has been shifted to a different fitness component (Williams *et al.*, 2006; Baudisch & Vaupel, 2012). In simultaneous hermaphrodites, resources may be directly reallocated from the female function to the male function or vice versa (Charnov, 1979; Schärer, 2009; Avise, 2011). Furthermore, simultaneous hermaphrodites are expected to allocate resources to their male and female sex functions in a size-dependent manner, where larger size leads to a greater investment in female function compared with male function (Charnov, 1982; Klinkhamer *et al.*, 1997; Schärer, 2009). Thus, a decline in reproductive function does not necessarily reveal physiological deterioration. It can result from a beneficial age-dependent allocation of resources rather than purely on the failure of selection to remove late-acting deleterious mutations or on delayed negative effects of alleles with beneficial effects early in life. To understand the basis of reproductive senescence, we need to distinguish between resource allocation and the deterioration of the ability to efficiently convert resources into offspring. We propose a framework to do this based on the analysis of age-related changes in reproductive investment (i.e. resource investment in each sex function), reproductive output (i.e. number of offspring), and reproductive efficiency (i.e. the ratio of reproductive output to reproductive investment).

To illustrate our point, we can imagine a simple model of a simultaneous hermaphrodite without senescence and with fixed sex allocation. As they grow, individuals invest more resources into male and female function (Fig. 1a), reproductive outputs increase in proportion (Fig. 1b), and reproductive efficiencies remain constant (Fig. 1c). Next, we imagine a model with a change in sex allocation, but with constant reproductive efficiency. Here, reproductive investment is increasingly female-biased (Fig. 1d) and reproductive outputs change accordingly (Fig. 1e). Although such a shift in allocation could be adaptive [e.g. if female reproduction benefits from an increase in size more than male reproduction (Charnov, 1982)], not all changes in allocation are adaptive; many factors can result in age-dependent changes in the ratio of energy invested into the male vs. female function. The important point is whether or not resources are invested into a given function, a question that is independent from whether allocation patterns result from adaptation or constraint. In a final scenario, allocation remains constant (Fig. 1g) but reproductive output declines with age (Fig. 1h); hence, reproductive efficiency declines. This decline cannot in principle be compensated for through any form of reallocation, because the energy is spent (albeit with increasing inefficiency) and cannot be reabsorbed. Such declines must therefore be interpreted as reflecting pure senescence. Although imagined here to be identical, the rate of decline in male and female reproductive efficiency may be very different for two main reasons: (i) one sex function may be a larger mutational target than the other, if, for example, it requires the expression of a larger number of specific loci; (ii) if sex allocation varies with age, the sensitivity of fitness to changes in reproductive efficiency will decrease faster for the earlier-expressed sex, resulting in a faster decrease in the strength of selection, and ultimately a faster senescence for that sex. Indeed, some combination of both a shift in sex

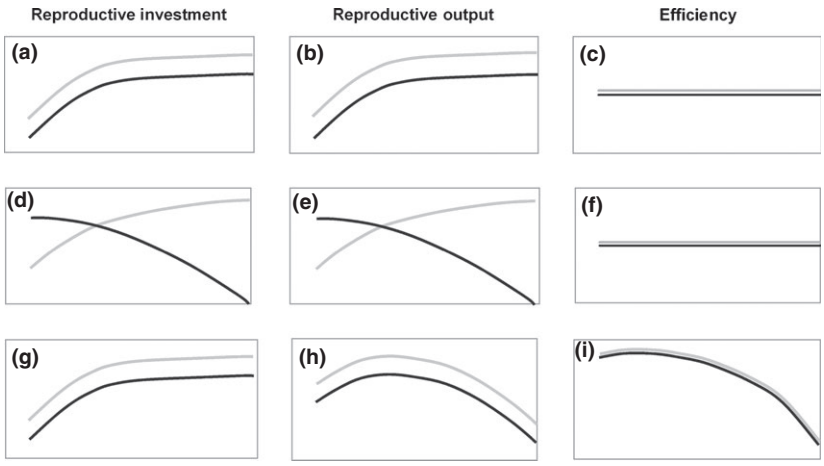

Fig. 1 Predicted patterns of male (black) and female (grey) reproductive investment (left), output (centre) and efficiency (right) as a function of age under three hypothetical scenarios: (i) no senescence and no change in sex allocation (a–c), (ii) no senescence but a change in sex allocation (d–f) and (iii) senescence but no change in sex allocation (g–i).

allocation and sex-specific declines in reproductive efficiencies may be highly probable. In sum, a decline in reproductive output with age can have multiple explanations including a decline in allocation and a decline in the ability to efficiently utilize resources; typically, both processes are labelled as senescence, but only the latter is purely deleterious.

Here, we report the result of an experiment designed to simultaneously measure reproductive investment and output (and hence reproductive efficiency) throughout an organism's entire lifespan. We do this for both male and female functions measured on the same individuals. We focus on organisms that are simultaneously hermaphroditic, where sex allocation can be viewed as plasticity and adjusted during an individual's life (Charnov, 1979, 1982; Schärer, 2009). However, aside from the fact that hermaphroditic individuals can allocate resources to both sex functions, our goal is to measure allocation to each function along with fitness gained through each function – a distinction that can likewise be made in organisms with separate sexes. To be clear, we do not endeavour to measure direct reallocation of resources from one sex function to another; doing so, while accounting for investment in growth, maintenance or any other trait, as well as loss of energy (e.g. via heat), is not feasible at this time or scale. Instead, we aim to measure, to the best extent possible, investment in male and female function along with reproductive output gained through each sex function. By examining how reproductive investment and output change with age, we aim to clarify the reasons why reproductive fitness declines with age. We argue that senescence, an unequivocally deleterious change in fitness with age, can only be demonstrated by measuring an allocation of resource into a particular function along with an age-related decline in performance through that function.

Materials and methods

We established a focal cohort of 23 families of the hermaphroditic, preferentially outcrossing freshwater snail *Physa acuta*. *Physa acuta* is a widespread, invasive freshwater snail (Bousset *et al.*, 2004) with a relatively short generation time (i.e. 6–8 weeks in the laboratory). It has been used repeatedly in studies of mating system and life-history evolution, including senescence of longevity (e.g. Escobar *et al.*, 2007, 2008; Auld, 2010; Pélissié *et al.*, 2012). Every 3 weeks, we sampled five individuals per family and assessed male and female copulatory activity and reproductive success. The focal individuals were older at each sample, but were provided with mates that were always the same age (see Fig. S1 for an overview of the sampling protocol).

Establishment and rearing of focal cohort and virgin mates

Approximately 150 adult snails were collected from a natural pond (Pataris I) near Montpellier, France. All snails were isolated, and we collected their eggs at 2-day intervals until we had obtained ~5 egg capsules from 50 adults (this occurred 4 day after isolation). Three weeks later (1 week after hatching), hatchlings were transferred to fresh boxes with a constant density of 5 per box, and finally, they were all isolated at 3 weeks post-hatching (shell length 2–3 mm), well before sexual maturity. This protocol promotes rapid development while ensuring virginity. At 6 weeks post-hatching (5–6 mm, near maturity), all individuals ($N = 867$) were uniquely marked with a combination of harmless dot of paint and a coloured, numbered tag (Ickowicz Apiculture; Henry & Jarne, 2007; Escobar *et al.*, 2007) and transferred to 3–l aquariums (30/aquarium). These marked individuals were considered to be the focal cohort for this study. To avoid con-

founding the effects of density (e.g. competition, mate availability) with age, density was restored weekly by replacing dead individuals with unmarked snails from stock cultures of the same population. The date of death was recorded for each marked individual. Individuals were moved among aquariums, and the haphazard placement of these aquariums was reorganized throughout the experiment. Starting at 7 weeks old, focal individuals were removed every 3 weeks for sampling and subsequently returned to experimental aquariums. Some marked focal individuals died before they could be used in the experiment. The number of snails sampled per family ranged from 21 to 64 with an average of 35.

Young, virgin mates were required for each sampling period (i.e. every 3 weeks). They came from extra families not used as focals and were handled as the focals except that they were maintained in individual boxes to ensure their virginity. New batches of eggs from stock cultures were collected every 3 weeks and handled as described above to produce virgin mate groups of constant age (7 weeks) for each sampling period.

Sampling periods

Focal individuals from each family were sampled every 3 weeks to assess male and female reproductive function. We obtained samples at ages 7, 10, 13, 16, 19, 22 and 25 weeks; note that under laboratory conditions, most individuals die before 6 months (Escobar *et al.*, 2008). During the first four sampling events, five individuals from each of the 23 families were sampled ($N = 115$). At 19 weeks, one family only had three individuals alive ($N = 113$). At 22 weeks, individuals were only available from 19 families and five individuals were only available for 11 of these families ($N = 77$). At 25 weeks, only 15 families were available and sample size varied from 1 to 3 per family ($N = 24$).

Each sampling period lasted 11 days (Fig. S1). On day 1, the focal (marked) snails were isolated in 75-mL containers. On day 4, their eggs were collected, counted and placed in fresh water. Surviving hatchlings were counted 15 days later (Escobar *et al.*, 2007; Auld, 2010). Reproductive adults were measured (maximum shell length measured to nearest 0.1 mm with digital calipers) and paired for 30 min with a 10-week-old (nonvirgin) unrelated mate and then reisolated for another 3 days. This duration of pairing in small (40-mL) containers allows snails to copulate and reciprocate if desired (Pélissié *et al.*, 2012). On day 7, eggs were collected to provide a second measure of fecundity and hatchling survival for the focal individuals, with the guarantee that each focal had had recent access to a young sperm donor. Subsequently (day 7), all individuals were paired with a 7-week-old virgin mate for 30 min during which we recorded all interactions between the two individuals [see Wethington & Dillon (1996), Jarne *et al.* (2010)]. We were able to unambiguously determine whether the focal was in the male (top) or female (bottom) position for any given sexual interaction. The timing of all events was recorded. If at the end of the 30-min observation period, copulation was still in progress, we continued observation to determine the length of the copulation. After observation, the two individuals were left together for 24 h to ensure ample opportunity for copulation and then separated. Focal individuals were then returned to the aquariums, and their mates were isolated in new containers for 3 days. Eggs laid by the mates were counted, and early survival of the offspring was assessed as described above. Because mates were virgin prior to pairing, their hatchlings represent the male siring success of the focals. Indeed, mature, virgin snails in similar conditions either lay 100% outcrossed eggs or none at all [Pélissié *et al.*, 2012; N.B. the only time selfing has been observed in comparable conditions is when the mates were much older and when a significant fraction of them had already started to lay selfed clutches before being paired (Janicke *et al.*, 2013)].

At each sampling period, 40 additional virgin mates were used as controls to standardize for unintentional fluctuation in laboratory rearing conditions. They were treated as the virgin mates except that they were paired with each other (20 pairs). Their copulations, fecundities and hatching success were recorded as well. One individual of each pair was marked for identification. This means that reproduction by the controls was their first outcrossing, which can enhance initial fecundity over same-aged nonvirgins (Tsitrone *et al.* 2003).

Male and female reproductive investment and output

Male reproductive investment was assessed as the time spent in the male copulatory position during the period of observation. It has always been problematic to measure allocation to the male function in hermaphrodites (Charnov, 1982; Schärer, 2009). Unlike the female function, the biomass of exported sperm is very small, hard to nondestructively measure and represents only a fraction of the male energetic expense. Individual snails move almost constantly, and male-acting individuals spend lots of energy on mating effort (e.g. mate searching, shell mounting and avoidance of rejection behaviours; Jarne *et al.*, 2010). Food searching and ingestion are also interrupted for the male-acting individual during copulation attempts. For these reasons, the total amount of time spent in the male position represents a reasonable surrogate of the male investment. Furthermore, research on other freshwater snails (Basommatophorans) has revealed that the stimulus for assuming the male position is the volume of fluid in the prostate gland (De Boer *et al.*, 1997; Koene *et al.*, 2000), another measure of male allocation. We used time in the male

position, including time in copulation, instead of only the time in copulation because (i) energetic costs are also spent during the precopulation phase and (ii) it is impossible to visually determine when sperm are actually being transferred during copulation. In addition, we are aware that we only sampled male activity for 30 min; however, after 3 day of isolation, most of the copulation attempts occur in the first 30 min (Pélissié *et al.*, 2012), and therefore, we buffer the error variance more efficiently by increasing numbers of individuals than by increasing the duration of each trial at the expense of sample size.

Male reproductive output was measured as the number of 15-day-old surviving offspring sired by each focal individual (i.e. laid by its mate). Individual focal snails that sired no offspring (i.e. zero eggs laid by the mate) spent on average 194 s (SE $\pm$ 25 s) in male position, whereas snails siring offspring spent on average 352 $\pm$ 26 s (81% more time). Thus, time in male position is an indicator of successful insemination.

Female reproductive investment was measured as the total number of eggs laid by each focal individual. Female reproductive output was assessed as the number of surviving maternal offspring. In both cases, we used the average (including zeros) of the two 3-day samples.

Statistical analyses

We fit generalized linear models in R (R Core Team, 2012) to assess the effects of age on male and female reproductive investment and output. Male investment (time in male position) was modelled as a Gaussian variable. Female investment (number of eggs), as well as male and female reproductive output (number of hatchlings), were modelled under a Poisson distribution (log link). All models included the appropriate control mean as an offset (i.e. as a linear predictor with a coefficient of 1; Crawley, 2005), which effectively results in expressing variables relative to control means. The effect of age in these models was assessed with a deviance analysis (Crawley, 2005), that is, by comparing the deviance of the model before and after deletion of the age term. Deviance ratios, which are approximately *F*-distributed, were calculated to account for overdispersion (Crawley, 2005). Preliminary analyses indicated that including family and individual identity (nested within family) as random effects did not alter the results; therefore, they were dropped from the final models. Models that include these random effects are summarized in Table S1).

We also analysed the effects of age on male 'fecundity', that is, the number of eggs sired by focals – eggs that were laid by the virgin mates. Preliminary analyses revealed that in 34% (231/675) of cases, the virgin mate did not lay any eggs, which reflects a failure by the focal individual to copulate or provide adequate sperm. Thus, we analysed male fecundity in two ways: (i) we assessed the effects of age on all cases of nonzero fecundity, and (ii) we analysed the probability of reproducing (as a binary variable) as a function of age.

Following the analysis of male and female reproductive investment and output, we tested for an effect of age on the ratio of output/investment (i.e. reproductive efficiency) by testing for heterogeneity of slopes. We first constructed a model in which the linear effect of age on both variables (relative reproductive output and relative reproductive investment, measured on a logarithmic scale) was constrained to be the same and then a second model in which we allowed different slopes for the effect of age on each variable. By comparing the deviances of these two models, we tested whether the effect of age on investment and output was significantly different. A significant difference reveals heterogeneity of slopes in logarithmic scale and thus a change in the ratio of output to investment with age.

We explored the effects of individual size on male and female reproductive traits within each age class. No effects of size of either the focal individual or the mate were consistent across the entire experiment [results not presented; see also Hermann *et al.* (2009)]. Size is, however, necessarily correlated with age (Fig. S2) and has been predicted to affect gender–role preference in hermaphrodites (Charnov, 1982; Schärer, 2009). As such, we examined the relationship between size of the focal individual (and relative size of the focal, compared with its mate) on time spent in the male and female copulatory positions via Spearman correlations.

Lastly, we examined the effects of age on the survival rate of paternal and maternal offspring and decomposed offspring survival into hatching success and post-hatching survival. Because juvenile survival may be density-dependent, we tested for an effect of density (eggs per box) on each survival rate. We modelled the effects of age and density on: (i) the entire survival rate (i.e. number of juveniles alive at 15 days/number of eggs), (ii) the hatching rate (i.e. number of hatchlings/number of eggs) and (iii) the post-hatching survival rate (i.e. number of juveniles alive at 15 days/number of hatchlings). All transitions were modelled as binomial variables with a logit link function. As above, deviance analyses were performed to account for overdispersion. We also fit mixed models including family and individual (nested within family) as random effects. The inclusion of these random effects did not alter the age effects, and they were dropped from the final models; the models including random effects are summarized in Table S2.

Results

Individual survivorship declines with age (Fig. 2a), but importantly, this decline markedly accelerates between 17 and 21 weeks. When plotted on a log scale, this acceleration indicates an increased mortality rate with

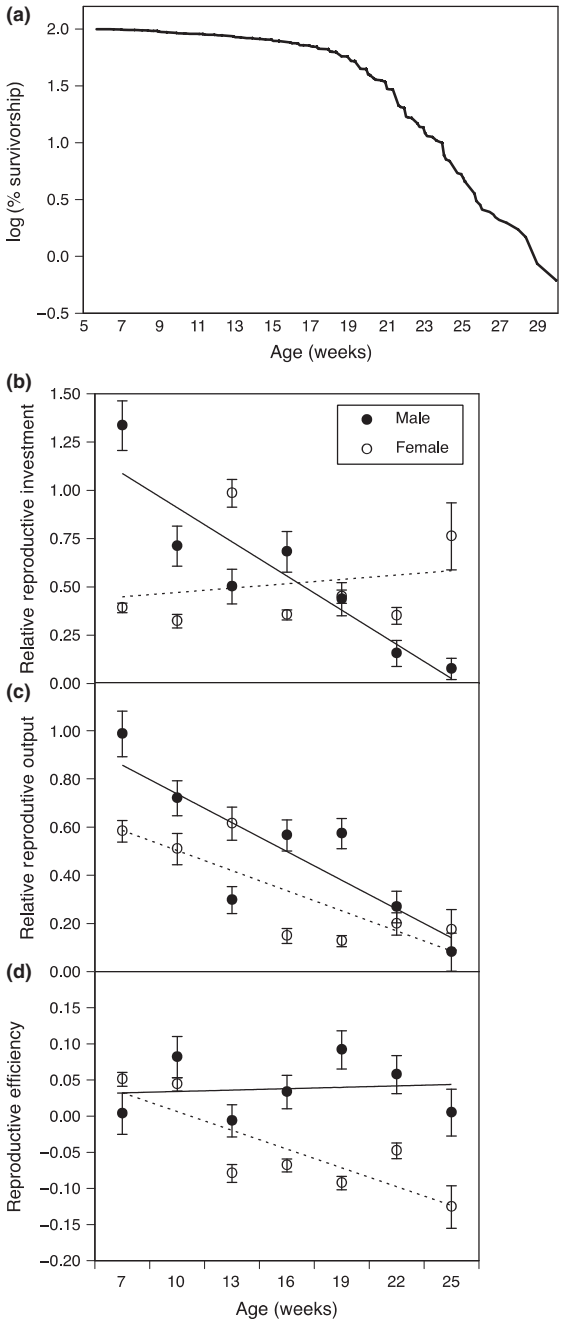

Fig. 2 Effects of age on survival (a) as well as male (solid) and female (dashed) relative reproductive investment, output and efficiency in *Physa acuta* (b–d). (a) Age-specific survivorship is plotted on a log scale to illustrate the acceleration of mortality with age. (b) Reproductive investment in male function is the time spent in the male position. Reproductive investment in female function is the number of eggs. (c) Reproductive output through male (female) function is the number of surviving offspring sired (laid) by a focal individual. Data are relativized by control means. (d) Reproductive efficiency is the difference between log-transformed reproductive output and investment. All values are means ± 1 SE. Raw data are plotted in Fig. S5.

age (i.e. actuarial senescence), as previously observed in this species (Escobar *et al.*, 2008). Individual reproductive investment (via both male and female functions) was not correlated with longevity (Fig. S3).

Female reproductive investment (i.e. the number of eggs laid) did not change with age, (Table 1, Fig. 2b), but female reproductive output (i.e. the number of maternal offspring produced) declined with age (Fig. 2c), indicating a decline in female reproductive efficiency with age (Fig. 2d). The negative effects of age on female reproductive output remain significant even after discarding cases where female reproductive investment was null ($F_{1,472} = 92.25$, $P < 0.001$). Senescence of female reproductive function was clearly evident in our analysis of juvenile survival rates (Table 2, Fig. 3b). Specifically, overall offspring survival was negatively affected by maternal age, as were its two components – hatching and post-hatching survival. Density had a weak, positive effect on the survival of maternal offspring, demonstrating no evidence for competition among juveniles. Thus, female function showed clear evidence for senescence at multiple stages.

Male reproductive investment (i.e. the amount of time that individuals spent in the male copulatory position) declined almost linearly with age (Table 1; Fig. 2b). This decline in male investment was matched by a decline in male reproductive output (i.e. the number of paternal offspring produced; Fig. 2c). When we removed instances where male fecundity was null (i.e. when the mate previously paired with a focal male failed to reproduce at all), the negative effect of male age remained highly significant ($F_{1,374} = 37.7$, $P < 0.001$). Further, the probability of successfully stimulating the mate to produce eggs (vs. no eggs) declined significantly with male age ($F_{1,605} = 28.7$, $P < 0.001$). As both investment and output decline with age, there was no overall trend for male reproductive efficiency with age (Fig. 2d), which indicates that the decline in male reproductive output with age largely stems from a decline in investment. However, we observed negative effects of male age on the overall survival rate of offspring (Table 2, Fig. 3). This survival rate was also slightly lower in larger clutches, perhaps a result of competition among newly hatched juveniles. However, when we decompose the overall survival rate of offspring, it is clear that male age had a negative effect on hatching. Further, the negative effects of density cannot explain this result as they only affect post-hatching survival. Thus, declines in male reproductive output with age can be attributed to both a decline in allocation and a decrease in efficiency.

The decline in time spent in the male position with age is also seen when time in male position is plotted as a function of size ($\rho = -0.54$, $P < 0.001$; Fig. 4); the relationship between size and age is plotted in Fig. S2. Simultaneously, time spent in the female copulation position increases with age, but this increase is not statistically significant.

Table 1 Results of deviance analyses on generalized linear models fit to assess the effects of age on male and female reproductive investment, output and efficiency. For the latter, we tested for heterogeneity of slopes in the age effect on investment and output (see text); an effect of age on efficiency is demonstrated by a significant Variable*Age interaction. The Term column shows the term that was deleted from the more complete model. Deviance ratios were calculated based on the d.f. and deviance explained by the more complete model and the model without a given term. *F*-ratios were calculated to account for overdispersion.

Gender	Variable	Term	Estimate	Explained deviance	*F*	d.f.	*P*
Male	Investment	Age	−0.103	42673	68.68	1,598	***
	Output	Age	−0.068	889	42.78	1,605	***
	Efficiency	Difference in slopes	0.039	292	0.91	1,1203	NS
Female	Investment	Age	0.003	4	0.23	1,673	NS
	Output	Age	−0.101	960	87.50	1,673	***
	Efficiency	Difference in slopes	−0.107	1057	67.38	1,1346	***

P-values: NS: $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 2 Results of deviance analyses on generalized linear models fit to assess the effects of age and density on male and female reproductive output. For each gender role, we tested the effects of age and density on the overall survival rate of juveniles controlling for the number of eggs [i.e. eggs (E) → juveniles (J)]. This overall survival rate was then decomposed into the hatching rate [i.e. E → hatchlings (H)] and the post-hatching survival rate (i.e. H → J).

Gender	Transition	Effect	Estimate	Explained deviance	*F*	d.f.	*P*
Male	E → J	Age	−0.03	81.5	3.79	1,373	*
		Density	−0.01	186.6	8.68	1,373	**
	E → H	Age	−0.05†	75.2	6.74	1,369	**
		Density	(0)	5.8	0.52	1,369	NS
	H → J	Age	(0)	0.4	0.02	1,367	NS
		Density	−0.01	160.5	7.39	1,367	**
Female	E → J	Age	−0.19	3931	233.6	1,652	***
		Density	0.01	71	4.22	1,652	*
	E → H	Age	−0.22	4481	233.7	1,651	***
		Density	0.01	116	6.05	1,651	*
	H → J	Age	−0.13	1569.7	109.9	1,625	***
		Density	0.01	119.4	8.36	1,625	**

P-values: NS: $P > 0.05$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.
†Model fit with quadratic age term also significant (estimate = −0.01, $F_{1,369} = 5.24$, $P = 0.02$).

Discussion

Under these experimental conditions, both sex functions showed a marked decline in reproductive output with age, results that would classically be interpreted as senescence. These declines were coincident with a rapid decline in survivorship (Fig. 2). However, individual reproductive investment was not related to survival (Fig. S3). Taken together, this indicates that, on average, individuals did not experience any survival benefit from reduced reproductive investment. Reproductive efficiency did not significantly change with age for the male function (Table 1); however, we observed a decline in the hatching rate of eggs sired by old males (Fig. 3c). The latter did not turn into a significant decrease in efficiency with age because the hatching rate decreases only slightly and the effect is restricted to very old males (22–25 week old). In contrast, for the female function, a strong decline in hatching rate was observed and started early (10–13 weeks), which resulted in a steady decline in reproductive efficiency throughout the reproductive life. Reproductive output for both sex functions declined considerably and steadily throughout the lifespan. These results are coherent when viewed through the lens of reproductive investment, as male and female outputs decrease for different reasons. The primary reason for the decline in male reproductive output is decline in male investment (efficiency being roughly constant). The primary reason for the decline in female output is a gradual decline in reproductive efficiency, female investment being relatively static throughout life. Our measures of male and female investment are of course imperfect, and future studies should endeavour a more detailed evaluation of the energy spent into the male and female functions. For example, we measured female investment by counting eggs, but female investment per egg may also vary with age (Fischer *et al.*, 2006). Considering that from 7 to 25 weeks, female efficiency and hatching rate declined to < 20% their original value, whereas the

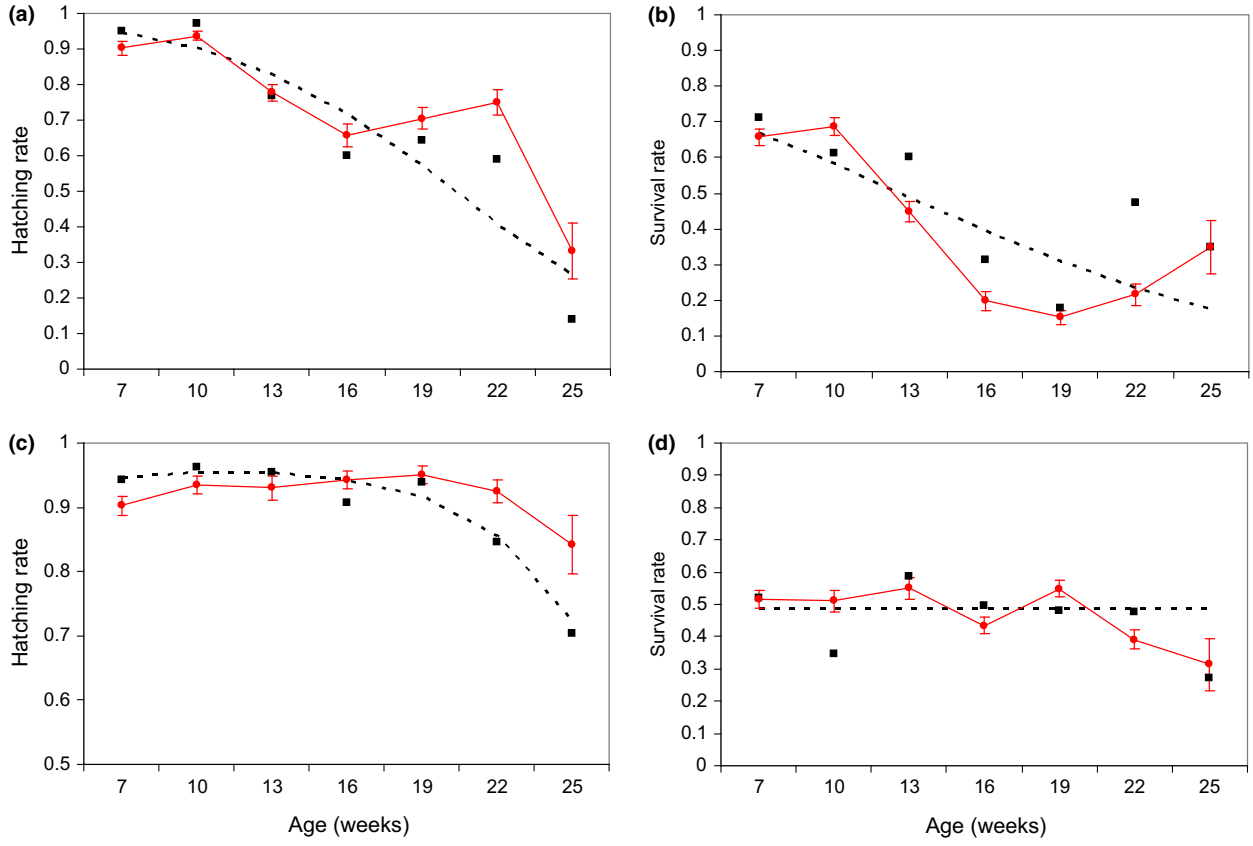

Fig. 3 Age-specific expected (dashed lines) and observed (symbols) hatching rates and survival rates for eggs laid through female (a, b) and sired through male (c, d) function in *Physa acuta*. Hatching rate (a, c) is the number of hatchlings divided by the number of eggs; Survival rate (b, d) is the post-hatching survival rate of juveniles (number alive at 15 days divided by the number of hatchlings). Expected values were estimated following the models reported in Table 2 for an average individual assuming constant density and average control values. The expected values for male hatching utilize the model with a quadratic effect of male age. Observed values (black symbols) were likewise estimated assuming constant density and average control values. Red values show the raw values of each variable (± 1 SE) not corrected for control values or density effects.

number of eggs increased by ca. 10% during the same time, we can safely exclude that the decline in female reproductive output could be matched by a decrease in investment, even if the later accounted for egg size (this would require a reduction in egg size to < 20% their initial size, which we would have certainly noticed, as we always counted control eggs from young individuals at the same time as eggs laid by focals). So, overall, the conclusion that female reproductive efficiency declines can safely be maintained. However, further research examining female reproductive investment at a more mechanistic level (e.g. measuring the volume and nutritional components of individual eggs) would definitely be welcome to provide more precise estimate. For the time being, our main point is that if we had not measured reproductive investment and output, we would not be able to distinguish when and how age-related declines in reproductive performance occur.

While demonstrating an age-related decline in investment in one function does not require a demonstration of resource reallocation into a different function, our data do provide some insights into how allocation patterns change with age (and size). Concurrent with declining survivorship, larger individuals reduced investment in male copulatory behaviour and increased their time spent in the female copulatory role. This does not conclusively demonstrate resource reallocation from the male role to the female role with age, but it is consistent with theory and empirical observations (in this species and others) that there is a shift from male to female behaviour as size increases (DeWitt, 1996; Schärer, 2009). As such, the proportion of energy allocated to the male function decreases with age (i.e. a change in sex allocation). Collectively, our data demonstrate that both changes in resource allocation and sex-specific declines in reproductive efficiency contribute to the sex-specific decline in reproductive output with age.

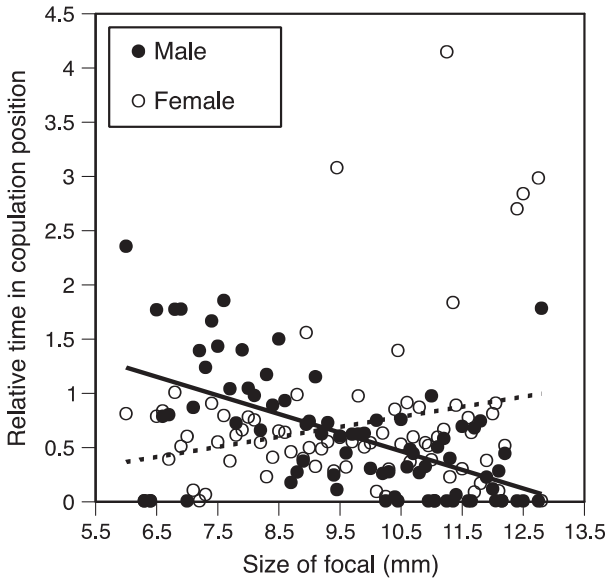

Fig. 4 Relationship between the size of the focal individual (maximum shell length measured just prior to copulation observation) and the time spent in male (solid black) and female (open symbols, dashed line) copulation positions. Times in copulation position are relative to control pairs (see text). The relationship is significantly negative for male position and positive, but not significant for the female position. Data are plotted as means for each size (to the nearest 0.1 mm).

Resource reallocation and reproductive senescence are not mutually exclusive sources of declining performance with age; senescence may decrease the ability to allocate resources to reproduction, to reallocate resources from one fitness component to another or simply to gather resources altogether. However, by labelling any and all declines in reproduction with age as 'senescence', as is generally done in senescence studies (Bonduriansky *et al.*, 2008; Baudisch & Vaupel, 2012), we can lose track of the different levels at which age-related declines may occur. In reality, it is extremely difficult (if possible) to measure resource investment in all functions (including loss) in the same units and therefore to know whether the total resource investment in them increases or decreases. Without any means to evaluate the change in total investment into male and female function with age, the idea remains untestable. On the other hand, a decline in reproductive efficiency, that is, in the fitness reward per unit energy invested, can unambiguously be labelled senescence because the energy cannot be reinvested into something else once it is spent in the form of eggs or male behaviour. Below, we discuss and compare the evidence for senescence in both male and female functions and how these observations are likely related to what occurs under natural conditions.

While declines in offspring fitness that can be attributed to maternal age have been documented in a variety of species (e.g. Charmantier *et al.*, 2006; Wilson *et al.*, 2007), examples of the negative effects of paternal age on offspring fitness are comparatively rare, especially in species without any form of parental care (but see Ducatez *et al.*, 2012). In one comparable study, Priest *et al.* (2002) found substantially negative effects of maternal age, yet weak effects of paternal age, on offspring survival in *Drosophila melanogaster*. In our experiment, the decline in hatching success with male age demonstrates that, as individuals age, the sperm (or associated seminal fluid) they transfer to their partners is in some way inferior to what is transferred earlier in life. It is known that individuals in this species are capable of storing sperm (Wethington & Dillon, 1991; Jarne *et al.*, 1993), and thus it is not clear whether (i) these old individuals are fertilizing eggs with sperm that are created late in life and may be inferior because they are produced from, for example, old germ cells, (ii) these individuals are using old (stored) sperm that have begun to senesce inside the paternal snail or (iii) the production of seminal fluid in old snails is somehow inferior and interacts negatively with the female reproductive system of the partner. That is, we cannot distinguish whether declines in hatching success result from pre- or post-meiotic sperm senescence (Pizzari *et al.*, 2007). Indeed, we know relatively little about the reproductive dynamics in snails (Geraerts & Joosse, 1984; Jarne *et al.*, 2010). It is usually assumed that sperm are typically produced continuously and degraded in the seminal vesicles, the turnover being on the order of a few days. Thus, premeiotic sperm senescence may be more likely, but future work to evaluate these options is clearly needed. In sum, although offspring hatching success is negatively affected by male age, the decline is minor and quite late in the lifespan relative to the decline in female function which explains why the overall decline in male reproductive efficiency with age is not statistically significant.

Our experiment assessed the effects of age on male and female reproductive function under laboratory conditions, which represent a simplified and to some extent unnatural set of circumstances; namely, our protocol featured no-choice pairings where intrasexual competition for access to mates was absent (i.e. no male–male competition), except for the fact that both members of the pair may prefer to assume the same gender role. Under natural conditions, episodes of low density do occur, but competition, namely male–male competition (including sperm competition within the recipient's female tract), may be important for determining which individuals gain successful copulations (Pélissié *et al.*, 2012). Senescence is, of course, likely to be context-dependent. Male efficiency may have remained high in our experiment because males were not in competition and only had to give enough sperm

to fertilize females – a condition that might change substantially with male–male competition. It is widely observed that allocation to male function increases when there are more mates available (Schärer, 2009), and as such, shifts in resource investment into male function with age are likely to be influenced by density. This is clearly an important subject for further experiments. Indeed, the number of copulations might be more important when there are several individuals in competition for fertilization (Pélissié *et al.*, 2012). Under more natural conditions, where male–male competition is likely to be prevalent, the decline in male function with age may be even more rapid. Further, our results may be to some extent conservative in that the best surviving individuals in our experiment most likely represent a nonrandom sample of the individuals present at the beginning of the experiment; if sex allocation is somehow dependent on condition, there is a risk that very old individuals may differ in the allocation patterns from the initial stock (Cam *et al.*, 2002; van de Pol & Verhulst, 2006). Future studies that evaluate these patterns under more natural conditions, including other levels of resource availability (e.g. to alter condition or sex allocation), are needed. There is nonetheless a trade-off between laboratory studies that present the opportunity for control over many parameters (e.g. the present study and work on, e.g. *Drosophila*; Priest *et al.*, 2002) and studies under more natural conditions (e.g. Charmantier *et al.*, 2006; Nussey *et al.*, 2009).

It is striking and to some extent paradoxical that the function whose efficiency declines faster (i.e. female function) is not the function to which allocation decreases with age. Indeed, allocation to male function decreased sharply with age, whereas allocation to female function, at least in terms of the number of eggs, stayed constant (or slightly increased, most likely reflecting size-specific fecundity). Further, the decline in female efficiency commences prior to the decline in survival, whereas the decline in male efficiency is only observed in older individuals. These results can be compared with previous work on this species where actuarial senescence was found to be consistent with the mutation-accumulation theory of senescence (Escobar *et al.*, 2008). Consequently, one may expect that senescence will occur as the result of relaxed selection on late performance (Promislow, 2003) and that selection may be all the more relaxed when the contribution of old age classes to population growth decreases. Starting from a no-senescence situation (i.e. constant efficiency with output proportional to investment in both sexes), if male and female functions were equivalent mutational targets, we would expect faster senescence to evolve on male reproductive efficiency because, given the decline in male allocation, there is a lower relative contribution of old age classes to male fitness compared with female fitness (an effect that may be under-estimated in our experiment, as previously discussed). This is not the case; on the contrary, we observe much faster and larger senescence on female reproductive efficiency, suggesting that male reproduction is a much narrower mutational target than female reproduction. The female function may indeed represent a larger mutational target because of the large number of glandular components needed to produce eggs and egg capsules. In general, if a larger number of (unique) genes are involved in female function, this may increase the opportunity for mutation accumulation. In addition, sperm are much more numerous than eggs, such that even if a great majority of sperm is not functional, there will still be enough to fertilize all of the available eggs (e.g. intra-ejaculate competition may eliminate many low-quality sperm). The hypothesis of premeiotic sperm senescence (Pizzari *et al.*, 2007) and intra-ejaculate sperm competition may be supported by the fact that offspring hatching success only declines for very old males – it may require a large fraction of the sperm to be of poor quality for the effects to actually be expressed in the offspring. Comparatively, for females, there is no such excess in gamete production and the loss in reproductive output is proportional to damage. In this respect, decreasing the fertilization ability of one ejaculate by a certain amount would effectively require the accumulation of many more mutations than decreasing female output by the same amount. Importantly, as we are dealing with a simultaneous hermaphrodite, other attributes of the life history (growth, survival) are common between the sexes. Hence, the divergent age-related trajectories of reproductive efficiency likely stem from selection operating on sex-specific reproductive function.

Regardless of whether an organism is hermaphroditic or gonochoristic, a decline in reproduction with age may stem from both a shift in resource allocation and a decline in the ability to efficiently convert resources into offspring. Hence, we argue that progress can be made on understanding the process and evolution of reproductive senescence by clarifying the nature of declining reproductive performance with age (Baudisch & Vaupel, 2012). In an organism with separate sexes, this could be done, for example by documenting, for each sex, where reproductive function declines in the following chain of events: gamete production, behavioural interactions with a mate, copulation, post-copulatory interactions and, ultimately, offspring fitness. A reduction in investment at any one stage necessarily means subsequent stages will decline as well, but clarifying the mechanism is important for understanding senescence. In hermaphrodites, the presence of two sexual functions in the same body provides an additional opportunity for strategic allocation of resources (Schärer, 2009). To distinguish a shift in resource allocation from a decrease in resource conversion efficiency, both reproductive investment and output must be measured throughout the lifespan.

Here, we have taken advantage of studying growth, survival and reproduction in a hermaphroditic species where resources can be reallocated between male and female function. Future work that examines the extent to which resources are directly reallocated among various fitness components will be important to further elucidate the mechanisms underlying reproductive senescence in this and other species. In species with separate sexes, the situation may be simpler in that reallocation can only be between current reproduction and other traits. Previous work demonstrating faster reproductive senescence by one particular sex (e.g. Fox *et al.*, 2003; Bonduriansky *et al.*, 2008; Nussey *et al.*, 2009) might be reconsidered in this light. Lastly, future work that resamples the same individual hermaphrodites throughout their lifespan will facilitate an analysis of the trade-off between male and female reproductive function and how this trade-off changes with age. Our data suggest that this trade-off exists, but without the ability to test the strength of the genetic correlation across age, we cannot conclusively assess this trade-off.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 An overview of the sampling protocol (described in the text) that was followed at each sampling period (i.e. every 3 weeks).

Figure S2 Growth trajectories indicating the relationship between size (maximum shell length) and age throughout the experiment.

Figure S3 The relationships between age-specific reproductive investment in male (A) and female (B) functions and longevity.

Figure S4 Analogous to Fig. S3, the relationships between age-specific reproductive output (the number of surviving juveniles for male (A) and female (B) functions and longevity.

Figure S5 Raw (not relativized) data for male and female reproductive investment and output (*cf*. Fig. 2 in main text).

Table S1 Results of deviance analyses on generalized linear models fit to assess the effects of family and individual (nested within family) on the estimated age effects on male and female reproductive investment and output (compare with Table 1 in main text).

Table S2 Results of deviance analyses on generalized linear models fit to assess the effects of family and individual (nested within family) on the estimated age effects on male and female reproductive output (compare with Table 2 in main text).

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