

**Increase in multiple paternity across the reproductive lifespan in a  
sperm-storing, hermaphroditic freshwater snail**

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## Abstract

Polyandry is a common phenomenon and challenges the traditional view of stronger sexual selection in males than in females. In simultaneous hermaphrodites, the physical proximity of both sex functions was long thought to preclude the operation of sexual selection. Laboratory studies suggest that multiple mating and polyandry in hermaphrodites may actually be common, but data from natural populations are sparse. We therefore estimated the rate of multiple paternity and its seasonal variability in the annual, sperm-storing, simultaneously hermaphroditic freshwater snail *Radix balthica* for the entire duration of the reproductive lifespan. We also tested if multiple paternity was associated with clutch size or embryonic development. To obtain these data we measured and genotyped 60 field-collected egg clutches using nine highly polymorphic microsatellite markers. Overall, 50% of the clutches had multiple fathers, and both the frequency (20-93% of clutches) and magnitude of multiple paternity (mean 1.3-3.8 fathers per clutch) substantially increased over time, probably because of extensive sperm storage. Most multiply sired clutches (83%) had a dominant father, but neither clutch size nor the proportion of developed embryos per clutch was associated with levels of multiple paternity. Both the evident promiscuity and the frequent skew of paternity shares suggest that sexual selection may be an important evolutionary force in the study population.

## Introduction

Female matings with multiple males (i.e. polyandry) are common in a wide range of taxonomic groups, including amphibians (Zamudio & Chan 2008), birds (Griffith *et al.* 2002; Schmoll 2011), fishes (Coleman & Jones 2011), mammals (Soulsbury 2010), reptiles (Uller & Olsson 2008), social insects (Jaffé 2014), and plants (Pannell & Labouche 2013). Despite its commonness, historically polyandry has been considered a rare exception. Female gametes are larger and less numerous than male gametes (anisogamy), which was assumed to favor females that increase resource acquisition rather than the number of mating partners (Bateman 1948). As a consequence, the dependency of reproductive success on mating success (i.e. sexual selection) was predicted to be weaker in females than in males (Bateman 1948; Jones 2009; Henshaw *et al.* 2016). A recent review indeed found this to be the case in numerous animal species (Janicke *et al.* 2016).

Although sexual selection may generally be stronger in males, females can still benefit from copulations with several partners (Clutton-Brock 2009). The benefits may be direct (Arnqvist & Nilsson 2000) or indirect (Zeh & Zeh 1996; Jennions & Petrie 2000; Bernasconi *et al.* 2003; Simmons 2005), and empirical evidence for both types of benefits has been accumulating (e.g., Simmons 2005). At the same time, the optimal number of male mating partners is likely to be also influenced by costs of mating (e.g., Nahrung & Clarke 2007; Hoffer *et al.* 2010; Lange *et al.* 2012; Toft & Albo 2015). Notably, many of these costs may also be incurred when copulating repeatedly with the same male, potentially confounding estimates of the adaptive value of polyandry (Simmons 2005).

In view of the multitude of potential factors influencing the costs and benefits of polyandry (and similarly, polygyny), it is not surprising that the strength of sexual selection can be quite variable in both space and time. In males, the opportunity for sexual selection can vary considerably between different species of animals (sexual selection in females was not reviewed: Moura & Peixoto 2013). However, the strength of sexual selection can also vary substantially among (e.g., Mobley & Jones 2007; Mobley & Jones 2009) or within natural populations of a single species. In the latter case, variation has been found both among (Serbezov *et al.* 2010) and within reproductive seasons (McLain 1992; McLain *et al.* 1993; Wacker *et al.* 2014). Intraspecific variation in the strength of sexual selection has been shown to be associated with the operational sex ratio (Mills *et al.* 2007; Fitze & Le Galliard 2008; Aronsen *et al.* 2013; Wacker *et al.* 2014), population density (e.g., McLain 1992; Aronsen *et al.* 2013) and food availability (Janicke *et al.* 2015). Given its potential volatility, point estimates of the strength of sexual selection in a population may risk to miss important dynamics, especially in species that mate and reproduce repeatedly within a single reproductive season (Wacker *et al.* 2014).

Hermaphroditic species were long thought to be exempted from the operation of sexual selection, as in such species male and female roles are unavoidably intertwined (Darwin 1871, p 269), but during the last four decades, this view has been challenged (Charnov 1979; Michiels 1998; Michiels 1999; Leonard 2005, 2006; Anthes *et al.* 2010). Many hermaphroditic animals have been shown to mate multiply, mostly under laboratory conditions (e.g., Jordaens *et al.* 2007), but in a few cases also in the field (e.g., Anthes *et al.* 2006). Moreover, in the majority of studied hermaphroditic species, multiple paternity within broods or clutches, providing a minimum estimate of the degree of polyandry in a population (Dunn & Lifjeld 1994; Griffith 2007; Uller &

Olsson 2008; Anthes *et al.* 2016), turned out to be common (e.g., Angeloni *et al.* 2003; Pongratz & Michiels 2003; Henry *et al.* 2005; Proestou *et al.* 2008; Lorenzi *et al.* 2014; Janssen & Baur 2015). Finally, in two species of simultaneously hermaphroditic freshwater snails sexual selection has been measured explicitly, and proved to be quite similar in strength and direction to corresponding values of gonochoric species (Anthes *et al.* 2010; Péliissié *et al.* 2012).

Nevertheless, not much is known about multiple paternity in natural populations of hermaphrodites, and even less about its variability between and within populations. Numbers of matings and the degree of multiple paternity have often been assessed using laboratory mating trials, which not only alter the conditions under which copulations and reproduction take place, but also predefine the number and identity of available mating partners (e.g., Pongratz & Michiels 2003; Evanno *et al.* 2005; Koene *et al.* 2009; Garefalaki *et al.* 2010; Kupfernagel & Baur 2011a; Lorenzi *et al.* 2014). In other studies, presumably inseminated adults have been collected in the field and then kept in isolation, and rates of multiple paternity have been measured among the progeny produced in the laboratory (e.g., Angeloni *et al.* 2003; Henry *et al.* 2005; Janssen & Baur 2015; Nakadera *et al.* 2017). Although making fewer assumptions than the laboratory mating trials, also these studies assume that multiple paternity rates measured in the laboratory reflect those in the natural field populations. This assumption would be problematic if, for instance, under natural conditions snails continued to accumulate sperm from consecutive matings with new partners, or if the laboratory setting altered patterns of sperm storage and usage.

To the best of our knowledge, to date only two studies have estimated multiple paternity in field-collected progenies of hermaphroditic animals. In the protandrous marine snail *Crepidula*

95 *fornicata* as many as 11 out of 12 clutches (91.7%) had multiple fathers (Proestou *et al.* 2008).  
96 By contrast, only 17 out of 130 broods (13.1%) were multiply sired in the simultaneously  
97 hermaphroditic barnacle *Tetraclita rubescens* (Kelly *et al.* 2012). Interestingly, in *T. rubescens*  
98 multiple paternity was noticeably more common in high-density (25.0%) than in low-density  
99 patches (4.1%), potentially indicating that hermaphrodites may show a similar association  
100 between population density and the strength of sexual selection as some gonochoric species (e.g.,  
101 McLain 1992; Aronsen *et al.* 2013). Differences in the level of multiple paternity between  
102 populations of hermaphrodites have also been found in two studies of laboratory-laid egg  
103 clutches (Kupfernagel *et al.* 2010; Nakadera *et al.* 2017). Both inter- and intraspecific variation in  
104 multiple paternity may thus be substantial in hermaphrodites, and obtaining more data from  
105 natural populations is essential. In addition, the frequency and extent of multiple paternity in a  
106 population may change during an individual's lifetime, something that has never been studied in  
107 a free-living hermaphrodite (but see Angeloni *et al.* 2003; Kupfernagel & Baur 2011b; Janssen &  
108 Baur 2015 for studies using lab-produced progenies of field-collected adults). The occurrence of  
109 temporal changes in multiple paternity is likely because many hermaphroditic species have, just  
110 like numerous gonochorists, the ability to store, mix and potentially choose between received  
111 sperm from earlier matings (e.g., Cain 1956; Madsen *et al.* 1983; Vianey-Liaud *et al.* 1989;  
112 Nakadera *et al.* 2014).

113 We therefore assessed variation in rates of multiple paternity during the entire reproductive  
114 season in a natural population of the annual, simultaneously hermaphroditic freshwater snail  
115 *Radix balthica*. Even though this species is self-compatible (Pfenninger *et al.* 2011; Haun *et al.*  
116 2012), selfing is very rare in the study population (Bürkli *et al.* 2017). Copulation is unilateral,

and both sperm digestion (shown in *Lymnaea stagnalis*: Koene *et al.* 2009) and sperm storage (shown in *Biomphalaria glabrata*, *Helisoma duryi* and *Lymnaea stagnalis*: Cain 1956; Madsen *et al.* 1983; Vianey-Liaud *et al.* 1989; Nakadera *et al.* 2014) are probably common, allowing for alternative individual-level sex-role decisions and patterns of fertilization. In the study population, snails have a single reproductive season of roughly three months, throughout which we collected clutches in which we estimated multiple paternity. We used nine highly polymorphic microsatellite loci as genetic markers and ensured that rates of multiple paternity were free of laboratory artifacts, the influence of father-specific offspring mortality, errors caused by incomplete sampling, and contamination with samples from other species. Moreover, we tested for a potential relationship between the degree of multiple paternity within an egg clutch and clutch size or embryonic development, respectively. We found that multiple paternity was widespread, but not associated with either clutch size or embryonic performance. Instead, multiple paternity strongly increased in both frequency and magnitude towards the end of the reproductive season, potentially indicating a change in the strength of sexual selection over time.

## Materials and Methods

### Study system

*Radix balthica* is a diploid, simultaneously hermaphroditic freshwater snail that lives in the shallow littoral zone of large water bodies throughout Europe (Cordellier & Pfenninger 2009; Pfenninger *et al.* 2011; Lawton *et al.* 2015). In Lake Zurich, Switzerland, *R. balthica* exhibits an annual life cycle. Snails hatch from eggs in spring and reach sexual maturity by the end of the year. The reproductive season lasts from March to May, during which snails may copulate repeatedly in both sexual roles and lay hundreds of eggs in distinct egg clutches. After this time, adult snails die and by late May the cohort is completely replaced by juveniles, resulting in non-overlapping generations and a generation time of roughly one year. In the laboratory, field-caught and thereafter isolated snails produce clutches at a rate of roughly one every 3.8 days. Embryonic development is direct and juveniles start to hatch about 17 days after oviposition (A. Bürkli unpublished data). Copulations are unilateral, but in laboratory settings pairs of snails often swap roles. The size of the population studied here (Uerikon, Lake Zurich) is fairly large (> 10'000 individuals) and has remained constant for almost 20 years (J. Jokela unpublished data).

*R. balthica* is characterized by high degrees of phenotypic variation, such as in shell shape (Pfenninger *et al.* 2006; Brönmark *et al.* 2011), genital anatomy (Schniebs *et al.* 2011) and mantle pigmentation (Ahlgren *et al.* 2013). This variation complicates taxonomic identification of *Radix* species (Pfenninger *et al.* 2006; Schniebs *et al.* 2011; Huňová *et al.* 2012; Lawton *et al.*

2015). We therefore used two sets of molecular markers to verify that only individuals of *R. balthica* were included in this study (for details see Bürkli *et al.* 2017). First, we sequenced the mitochondrial cytochrome oxidase subunit I (COI) gene, which was identified as suitable for species delineation in *Radix* (Lawton *et al.* 2015). We did this for 86 adult snails collected from the study population in the year before conducting this study. All but one of these snails (98.8%) proved to be *R. balthica*. Second, we tested all microsatellite loci used in this study on molecularly identified individuals of *R. ampla*, *R. auricularia* and *R. lagotis*, as well as on two more distantly related species (*Lymnaea stagnalis* and *Physa acuta*). All species showed characteristic and consistent patterns of locus-specific amplification failure and had fixed alleles at some of the amplifying loci. With this information we then screened all adult and embryo genotypes used in this study, but only found individuals of *R. balthica*.

## **Collection of egg clutches and adult snails**

We collected adult snails and egg clutches in Uerikon, Lake Zurich, using snorkelling equipment. Clutches were used for estimating rates of multiple paternity, while adult snails served as candidate parents in parentage analyses. Adults and clutches were collected repeatedly over the entire reproductive season of the year 2014 (Table 1). We took care to collect them from a relatively large area (approx. 900 m<sup>2</sup>).

Clutches were gently detached from boulders using a plastic spoon and transported to the laboratory at Eawag-Dübendorf, where they were placed in individual 40 ml plastic cups filled with aged tap water (room temperature 18°C). Clutches that were torn or showed other signs of damage were discarded because their clutch size could not be ascertained unequivocally. Every

second day clutches were inspected for signs of hatching. When hatching was imminent, but at the latest 18 days after collection, clutches were transferred to 1.5 ml plastic tubes and stored at -80°C. Later, clutches were thawed and the number of developed and undeveloped embryos was counted in each clutch using a dissection microscope. We considered fertilized eggs in which development was arrested at various stages to be ‘undeveloped embryos’. Unfertilized eggs were extremely rare (0.009% of eggs) and are also included in this category. We refer to clutch size as the sum of developed and undeveloped embryos.

Adult snails were collected by hand and transported in individual containers to the laboratory, where we measured shell length to the nearest 0.1 mm using a digital calliper. Snails were kept in individual 200 ml plastic cups filled with aged tap water and were fed *ad libitum* with organic lettuce. We used these snails for various behavioral experiments in the laboratory. Between 0 and 74 days after collection, we killed the snails in 70% ethanol and stored them at -80°C for later genotyping.

## **Genetic analysis**

We estimated rates of multiple paternity in a total of 60 clutches collected on four dates spanning the entire reproductive season ( $n = 15$  clutches per sampling date, see Table 1). As candidate parents for parentage analyses we genotyped  $\geq 49$  adult snails per sampling date, except for date 23.05.2014, when due to the end of the reproductive season only 23 adult snails could be found alive. At all dates, the adults were collected at the same time as the clutches. If a clutch had more than 15 embryos, we genotyped 15 randomly chosen embryos. If it had 15 or fewer embryos, we genotyped all of them. We also genotyped undeveloped embryos (2.9% of genotyped embryos,

and 3.5% of embryos present in all genotyped clutches). This was done to avoid a possible bias caused by an unequal representation of paternal genotypes among developed and undeveloped embryos. All samples were genotyped for ten microsatellite markers (GenBank Accession No. KX830983-KX830992) that were developed specifically for this population by Ecogenics GmbH (Zurich, Switzerland).

Subsequently, we screened all loci for the presence of null alleles using > 2000 multilocus genotypes of snails originating from the Uerikon population, replicate genotypes of 54 adult snails, and comparisons of mother and offspring genotypes in lab-reared snails. The screening process identified one locus with a non-negligible frequency of null alleles (locus Rb\_3, GenBank Accession No. KX830985), which we excluded from all analyses. The remaining nine loci showed high levels of allelic diversity. The number of different alleles per locus (mean  $\pm$  SD) was  $7.1 \pm 3.2$  within groups of 15 embryos, each sampled from a separate clutch on the same day, and  $7.6 \pm 3.0$  within groups of 15 randomly chosen adults caught on the same day. This represents 24-25% of the maximum possible level of allelic diversity (100% = 30 alleles per locus in 15 individuals). Accordingly, the probability that randomly drawn adult snails share an identical multilocus genotype (P(ID)unbiased from Waits *et al.* 2001) was extremely small on all four sampling dates (between  $1.8 \times 10^{-11}$  and  $2.7 \times 10^{-10}$ ). Statistical power in our dataset is thus sufficient for reliable parentage assignment. Details of markers, genotyping routines, repeatability tests and of the steps we took to identify and control for the distorting effect of null alleles can be found in Bürkli *et al.* (2017).

## **Rates of multiple paternity**

Parentage analyses were performed using COLONY version 2.0.5.9 (Jones & Wang 2010). COLONY implements a full-pedigree likelihood method to simultaneously infer sibship and parentage by using individual multilocus genotypes. It accommodates both hermaphroditic species and polygamous mating systems. As COLONY does not require previous knowledge of maternal genotypes but rather reconstructs them based on the offspring genotypes present in a clutch, paternity can be inferred from field-collected clutches with unknown mothers. These clutches represent fertilization events that are free from laboratory artifacts.

Parentage was inferred simultaneously for all clutches collected on the same date. The genotypes of adult snails from the same sampling date were entered into COLONY both as candidate mothers and fathers, and clutch identities were specified as maternal sibships. The true parents of a clutch can but do not need to be present among the sample of candidate parents. The parameters for COLONY reconstructions were set as female and male polygamy, with inbreeding, in a monoecious species, short length of run, full-likelihood analysis at medium precision, without updating allele frequencies and with no sibship prior. The probability that the correct mother or father is among the candidate parents was arbitrarily set to 0.25, knowing that the true probability will be substantially lower and that the chosen value is merely used as a starting point for iterations.

As parentage assignments can be distorted by both null alleles and genotyping errors (Dakin & Avise 2004; Hoffman & Amos 2005; Dabrowski *et al.* 2015), we included two types of locus-specific error rates in all parentage analyses (Jones & Wang 2010). We computed the “null allele error rate” from the frequency of genotypes with amplification failure (mean: 5.1%,

maximum: 8.2%; following Dabrowski *et al.* 2015), and a rate of other types of genotyping errors from the frequency of microsatellite peaks that could not be scored for other reasons (mean: 1.7%, maximum: 4.7%). All locus-specific error rates can be found in Bürkli *et al.* (2017).

For each embryo, COLONY identified the most likely parental genotypes. If a candidate parent was a good match, COLONY returned the assignment probability to this parent. If the true parents were not among the candidate parents, COLONY returned the inferred parental genotypes. In addition, each embryo was assigned a list of other embryos  $1 \dots i$ , including embryos from other clutches, that were potential full siblings, each with its own probability  $p_1 \dots p_i$  of being a full-sib of the focal embryo. To measure the reliability of inferred full-sib families, we first computed each embryo's mean assignment probability to its full-sibs, and then a grand mean of embryo-level means. Two additional quality measures for parentage assignments were provided by COLONY. The “inclusive probability” of a full-sib family denotes the probability that all analyzed members of this family are indeed full-sibs, while the “exclusive probability” denotes the probability that all members are full-sibs and that none of the other analyzed embryos are full-sibs belonging to this family.

In addition, the minimum number of fathers in a clutch was estimated for each clutch by simply counting the number of alleles at the most diverse locus in the embryos' gene pool, subtracting two (the mother's contribution), and dividing the resulting number in half. This number is a minimum estimate because it assumes that all parents are heterozygous, and that each parent has two unique alleles not shared by any other parent.

## **Completeness of sampling**

Ideally, all embryos present in a clutch would be genotyped, and consequently the number of fathers in a clutch would be known without error. Unfortunately, limited resources often call for a

somewhat less exhaustive approach. In this study, we decided to genotype 15 randomly picked embryos per clutch, irrespective of clutch size. We thereby keep the power for detecting fathers at a constant rate of  $x$  fathers per 15 embryos.

However, to know how often the true number of fathers in a clutch will be revealed by genotyping only a fraction of its embryos, we genotyped seven clutches as comprehensively as possible ( $91.4 \pm 5.2\%$  (mean  $\pm$  SD) of all embryos genotyped successfully, Table 2). For each of these clutches we computed the probability of detecting all fathers in a subsample of 15 embryos by randomly picking 15 embryos without replacement and then counting the fathers in this subsample ( $n = 1000$  iterations). These simulations showed that, by genotyping 15 embryos, the probability of finding all the fathers that sired a clutch was high (89-100%, mean 96.8%) in most clutches (6/7, Table 2). One clutch was an exception. In this clutch with 36 embryos, 27 were sired by father 1 (75%), 8 by father 2 (22.2%), and a single embryo by father 3 (2.8%). As a result, only 42.5% of the subsamples of this clutch revealed all three fathers, while another 57.4% revealed only fathers 1 and 2. This illustrates how fathers with very low representation in a clutch ( $< 5\%$ ) may go undetected.

## **Statistical analysis**

We used the most likely number of fathers that sired a clutch, estimated using COLONY, as our measure of multiple paternity. Where  $> 15$  embryos had been genotyped from a clutch and the estimated number of fathers was  $> 1$ , we identified the likely number of fathers present in exactly 15 randomly chosen embryos ( $n = 1000$  iterations) and used this non-integer value in all analyses ( $n = 11$  multiply sired clutches with  $> 15$  genotyped embryos).

278 There were significant differences in variance across sampling dates in the number of fathers per  
279 clutch, in clutch size, and in the number of developed embryos per clutch (see Results). The  
280 homogeneity of group variances was assessed using nonparametric Fligner-Killeen median tests,  
281 which are robust against departures from normality (Conover *et al.* 1981; Ware *et al.* 2013). To  
282 test for a temporal effect in these variables, we therefore fitted generalized least squares models  
283 that allow for heteroscedasticity using the function “glS” in the R package “nlme” (Pinheiro *et al.*  
284 2016). In these models only sampling date (a continuous variable going from 1 to 79) was  
285 included as a predictor. We compared models in which variance changed continuously through  
286 time to models in which variance differed randomly between sampling dates, and found the latter  
287 models to perform better, both based on AIC values and diagnostic plots. For the number of  
288 fathers per clutch we ran an additional model including squared sampling date as a second  
289 predictor, but this resulted in higher AIC values and was therefore omitted. We also fitted two  
290 analogous models in which either the minimum number of fathers based on counting alleles or  
291 the number of fathers per genotyped embryo ( $1 \leq x > 0$ ) was the dependent variable. The  
292 relationship between sampling date and the proportion of developed embryos per clutch was best  
293 explained by a curvilinear model that included both sampling date and squared sampling date as  
294 predictors. Before fitting this model, we added ten to all sampling dates and log-transformed  
295 them to account for the slight asymmetry of the curved relationship.

296 To test for a potential relationship between the extent of multiple paternity and clutch size, the  
297 number of developed embryos per clutch, and the proportion of developed embryos per clutch,  
298 we added the number of fathers per clutch as an additional predictor to the models described  
299 above.

300 Shell lengths of field-caught snails were normally distributed (Shapiro-Wilk normality test:  $W =$   
301  $1.00$ ,  $p = 0.39$ ) and were analyzed using a simple linear regression with sampling date as the sole  
302 predictor. We added squared sampling date as a second predictor, but doing so did not improve  
303 the model fit significantly (ANOVA comparing the two nested models:  $F_{1, 1010} = 1e-04$ ,  $p = 0.99$ ).  
  
304 Unless stated differently, all analyses were performed using R v. 3.0.1 (R Core Team 2013).  
305 Values are given as mean  $\pm$  SD. In Figures 3 and 4 a small amount of noise was added to  
306 x-values to increase the visibility of individual data points.

## Results

### Quality of parentage assignments

Of the 1034 genotyped embryos included in this study, 90.2% were scored successfully at all nine loci, 98.2% at eight or more loci, 99.9% at seven or more loci, and 100% at six or more loci. Full-sib families inferred by COLONY had very high assignment probabilities. The mean assignment probability of an embryo to all its full siblings was  $99.5 \pm 0.02\%$  ( $n = 999$  embryos with full-sibs, Table 3). Only five embryos were assigned to their full-sibs with a mean probability of  $< 85\%$  (all  $\geq 61.2\%$ ). Accordingly, both inclusive and exclusive probabilities of inferred full-sib families were very high (Table 3). Seven out of 150 inferred parental genotypes (4.7%) matched some of the candidate parents collected simultaneously with egg clutches (Table 4). Moreover, in all clutches the most likely number of fathers, estimated by COLONY, was equal to or higher than the minimum number of fathers estimated by counting alleles (Table 4). All of this indicates that parentage assignments, and hence estimated rates of multiple paternity, are reliable.

### Multiple paternity in field-collected egg clutches

While none of the embryos genotyped for this study were self-fertilized (Bürkli *et al.* 2017), 30 of the 60 genotyped clutches (50%) had embryos sired by more than one father (Figure 1). The most likely number of fathers within a clutch ranged from one to nine, with an overall mean of  $2.1 \pm 1.5$  and a median of 1.5 fathers (Table 4). The level of multiple paternity within clutches varied significantly across sampling dates (F-K median  $\chi^2 = 13.7$ ,  $df = 3$ ,  $p = 0.0033$ ), with

variance increasing over time. Altogether, in 25 out of 30 multiply sired clutches (83.3%), a single dominant father sired > 50% of genotyped embryos (Figure 1). Consequently, the overall distribution of paternity shares within clutches was U-shaped, with an overabundance of both very rare fathers that sired only one out of 15 embryos (27.0%), and fully dominant fathers that sired all 15 embryos (21.5%, Figure 2). By contrast, fathers siring intermediate numbers of embryos (2-14) were relatively rare (51.6%).

### **Parents found in several clutches**

Twenty-two parental genotypes were found in more than one clutch (Table 5). In analogy to capture-mark-recapture studies, we consider these parents “recaptured” (see legend of Table 5 and Supporting Methods for details of how “recaptured” parents were detected). The phenomenon of “recaptured” parents gives several important insights into the studied mating system. First, four fathers were “recaptured” as mothers or vice versa (Table 5), showing that at least some snails reproduced in both sexual roles, thereby tapping their full potential as simultaneous hermaphrodites. Second, two snails were mothers and “recaptured” as mothers, i.e. they each were the mother of two genotyped clutches (Table 5). Apart from demonstrating the iteroparity of this species during its single reproductive period, these two pairs of clutches also revealed that sequential draws from a common allosperm reservoir yielded mostly (but not exclusively) the same fathers (see Supporting Results for details). And third, 17 fathers were “recaptured” as fathers (Table 5), usually in two clutches, but five fathers were each found in three and one father even in five clutches. As all the genotyped clutches these fathers sired had different mothers, this shows that the number of mating partners can be substantial also in the male role.

## Changes during the reproductive season

The most likely number of fathers per clutch almost tripled over the course of the breeding season (from  $1.3 \pm 0.6$  to  $3.8 \pm 1.8$ ,  $b = 0.029$ ,  $t = 5.03$ ,  $p < 0.0001$ , Figures 1 and 3a, Table 4). In other words, for every ten days that passed, a clutch gained 0.29 fathers on average. Using the minimum number of fathers per clutch did not change this result substantially (estimated by counting alleles,  $b = 0.013$ ,  $t = 5.72$ ,  $p < 0.0001$ ). A similar increase in the degree of multiple paternity over time was evident when looking at the mean number of fathers per genotyped embryo ( $b = 0.002$ ,  $t = 5.08$ ,  $p < 0.0001$ ).

Similarly, the proportion of multiply sired clutches increased from 20.0% at the start of the breeding season to 93.3% at its end (Figure 1, Table 4). As 95% confidence intervals for these estimates do not overlap, it is unlikely that this increase was a sampling error (0.04-0.48 vs. 0.68-1.00; Zar 1996). Given that both the magnitude and frequency of multiple paternity increased over time, 74.1% of all dominant fathers were found on the first two sampling dates, while 94.1% of all rare fathers (siring only 1 out of 15 embryos) were found on the last two sampling dates (Figure 2).

During the same time period, clutch size decreased almost by 50% on average, dropping from a mean of  $64.0 \pm 22.9$  eggs per clutch to  $33.0 \pm 14.2$  eggs per clutch ( $b = -0.42$ ,  $t = -7.33$ ,  $p < 0.0001$ , Figure 3b). Clutch size and the number of developed embryos were highly correlated ( $r = 0.98$ ,  $p < 0.0001$ ), and thus the same temporal decrease was evident in the number of embryos per clutch that developed normally ( $b = -0.37$ ,  $t = -6.69$ ,  $p < 0.0001$ ). Both clutch size (F-K median  $\chi^2 = 18.1$ ,  $df = 6$ ,  $p = 0.0060$ ) and the number of developed embryos (F-K median  $\chi^2 = 17.5$ ,  $df = 6$ ,  $p = 0.0077$ ) showed significant differences in variance across sampling dates. Meanwhile, the

proportion of developed embryos in a clutch slightly but significantly decreased towards the middle of the breeding season and increased again towards the end (adjusted  $R^2 = 0.06$ ,  $F_{2, 198} = 7.41$ ,  $p = 0.0008$ , linear effect of sampling date:  $b = -0.38$ ,  $t = -3.50$ ,  $p = 0.0006$ , quadratic effect of sampling date:  $b = 0.06$ ,  $t = 3.64$ ,  $p = 0.0004$ , Figure 3c). This effect remained significant also after excluding the two clutches with < 50% developed embryos (adjusted  $R^2 = 0.12$ ,  $F_{2, 196} = 14.72$ ,  $p < 0.0001$ , linear effect of sampling date:  $b = -0.30$ ,  $t = -4.56$ ,  $p < 0.0001$ , quadratic effect of sampling date:  $b = 0.05$ ,  $t = 4.82$ ,  $p < 0.0001$ ).

Finally, over the course of the reproductive season the shell length of field-caught snails increased from  $13.2 \pm 1.7$  mm to  $15.7 \pm 1.7$  mm ( $b = 0.04$ ,  $t = 12.21$ ,  $p < 0.0001$ , Figure 3d). This shows that somatic growth did not come to a stop once snails began to reproduce, indicating that the observed decline in clutch size did not co-occur with a predominance of relatively small snails in the second half of the reproductive season.

### **Relationship of multiple paternity with clutch size and embryonic development**

The number of fathers in a clutch was not a significant predictor for clutch size ( $b = 0.31$ ,  $t = 0.16$ ,  $p = 0.87$ , Figure 4a), the number of developed embryos ( $b = -0.22$ ,  $t = -0.12$ ,  $p = 0.91$ ), or the proportion of normally developed embryos ( $b = -0.01$ ,  $t = -1.34$ ,  $p = 0.19$ , Figure 4b). These models included sampling date as a second predictor, which accounted for significant amounts of variation in response variables in all three models (all  $p \geq 0.0021$ ). Without sampling date as a predictor, the number of fathers per clutch significantly predicted both clutch size ( $p = 0.0303$ ) and the number of developed embryos ( $p = 0.0240$ ), but not the proportion of developed embryos

392 ( $p = 0.46$ ). However, these effects are driven solely by temporal changes in clutch size and  
393 therefore reflect spurious covariance between predictors, as shown when running separate models  
394 for each of the four sampling dates (all  $p \geq 0.28$ ).

## Discussion

The degree of multiple paternity and its link to fitness components are important evolutionary parameters because they help elucidate both the potential role of sexual selection in a population and the full range of reproductive strategies available to individuals. Such insights are of particular interest in populations of self-compatible hermaphrodites, as these species command an exceptionally extensive repertoire of reproductive options. In this study, we investigated whether field-collected egg clutches laid by an annual, self-compatible, hermaphroditic freshwater snail had multiple fathers, whether levels of multiple paternity changed throughout the reproductive season, and whether multiple paternity was associated with either variation in clutch size or in embryonic development. We found multiple paternity in half of the genotyped egg clutches, with clutches having 2.1 fathers on average. Intriguingly, both the frequency and magnitude of multiple paternity significantly increased during the reproductive season. The season started with 20.0% multiply sired clutches and 1.3 fathers per clutch, and ended with 93.3% multiply sired clutches and 3.8 fathers per clutch. However, neither clutch size nor the proportion of normally developed embryos was associated with levels of multiple paternity. These results suggest that the strength of sexual selection potentially changes throughout the season, but they also illustrate the difficulty of evaluating possible effects of multiple paternity in the absence of knowledge about the lifetime reproductive success of individuals and about variation in their mating strategies across the full reproductive lifespan.

Estimating levels of multiple paternity in natural populations is notoriously challenging. We controlled for several possible sources of error that are often problematic for these types of

studies, and are thus confident that our data accurately represent levels of multiple paternity in the study population. We collected clutches in the field to have a representative sample of natural fertilization events unaffected by laboratory artifacts. Pre-genotyping mortality, and with it a possible bias due to father-specific differences in offspring survival, was reduced to near-zero by genotyping unhatched and also undeveloped embryos. We also quantified the potential error caused by limited sample size and found that only very rare fathers (per-clutch paternity share of  $< 5\%$ ) may have remained undetected. Our polymorphic, purpose-made molecular markers provided high statistical power for a reliable assignment of paternity, and allowed us to control and remove the possible bias caused by null alleles. We analyzed parentage using COLONY (Jones & Wang 2010), a software well-known for yielding highly accurate parentage assignments (Jones *et al.* 2010; Harrison *et al.* 2013). Finally, we expect that our estimates reflect the multiple paternity rate for the entire reproductive lifespan as this *R. balthica* population exhibits an annual life cycle and we collected data throughout the reproductive season.

Studies of multiple paternity in natural populations of hermaphroditic animals are rare. To the best of our knowledge, only two such studies exist to date. These studies report both low and high frequencies of multiply sired broods (13.1% in the barnacle *Tetraclita rubescens* vs. 91.7% in the marine snail *Crepidula fornicata*, Proestou *et al.* 2008; Kelly *et al.* 2012), suggesting that multiple paternity rates may be rather variable. Several studies have analyzed progenies of field-mated animals that were reproducing in the laboratory, assuming that, through sperm storage, lab-produced and field-collected progenies are comparable. For example, in the freshwater snail *Lymnaea stagnalis* 59% of clutches were multiply sired and clutches had a minimum number of 1.8 fathers on average (Nakadera *et al.* 2017). In *Physa acuta*, another

freshwater snail, clutches had a mean of 3.8 fathers (Henry *et al.* 2005), and in the terrestrial snail *Ariana arbustorum* 68.3% to 100% of clutches were multiply sired, and clutches had 4.0 to 6.1 fathers on average (Kupfernagel *et al.* 2010; Kupfernagel & Baur 2011b; Janssen & Baur 2015). Interestingly, our estimates of multiple paternity rates are roughly in the same range as these laboratory-based estimates. It is probably safe to conclude that multiple paternity is not necessarily rare in hermaphroditic animals and that laboratory and field estimates are not in strong contradiction. Nevertheless, to assess their similarity conclusively, both types of estimates would need to be obtained using individuals from the same population and ideally the same cohort.

The increase in multiple paternity towards the end of the reproductive period is a new and intriguing finding. Only a few studies have examined temporal trends in multiple paternity in hermaphrodites, all of them using laboratory-laid egg clutches. Neither in the marine snail *Aplysia californica* (Angeloni *et al.* 2003) nor in *A. arbustorum* (Janssen & Baur 2015) there was any evidence for such trends, although in *A. arbustorum* one study found more multiple paternity among adult than subadult snails (Kupfernagel & Baur 2011b). Also in gonochorists, data on within-season variation in multiple paternity are very scarce. In a natural population of the live-bearing fish *Poecilia latipinna*, the rate of multiple paternity was higher in fall than in spring (Trexler *et al.* 1997).

What could cause such a substantial temporal increase in multiple paternity in our study system? The most likely explanation is an increase in the number of mating partners contributing sperm to a snail's allosperm reservoir. Although not yet shown for *R. balthica*, sperm storage and its utilization later in time have been documented in several species of Basommatophoran freshwater

snails (Cain 1956; Madsen *et al.* 1983; Vianey-Liaud *et al.* 1989; Nakadera *et al.* 2014). We thus hypothesize that the number of fathers per clutch increases because snails habitually store and mix received sperm and only use it little by little. Sperm accumulation is also suggested as a potential cause of increasing rates of multiple paternity in the gonochoric fish *P. latipinna* (Trexler *et al.* 1997). Alternatively, increasing levels of multiple paternity could reflect an increase in mating frequency, but this scenario seems unlikely given the annual life cycle of *R. balthica* in Lake Zurich. In the second half of the reproductive season, adult population size declines steeply and eventually all adults have died by the end of May, which effectively and progressively restricts mating opportunities. Another hypothesis in accordance with the life cycle is senescence of snails. Late-reproducing snails would be forced to use sperm from multiple mates for fertilization if the amount and/or quality of received sperm declined as the season progressed, or if stored allosperm had degraded or became depleted.

A counterintuitive result of our study is that the temporal increase in multiple paternity was paralleled by a strong decrease in clutch size. As clutch size is positively correlated with body size in many Basommatophoran freshwater snails (e.g., *L. stagnalis*: Koene *et al.* 2007; *Radix lagotis*: Yu & Wang 2013), the observed decline in clutch size could stem from a shift in the fraction of egg-laying snails from large, fast-growing snails early in the season to comparatively small, slow-growing snails later in time. However, this explanation is unlikely given that we found adult snails to be largest on the last sampling date. Smaller clutches at the end of the season could also arise from differences in the packaging of eggs that are produced at a constant rate, resulting in many small instead of few large clutches. Alternatively, small clutches may be a sign of reproductive senescence. A decrease in dry weights of egg clutches, numbers of

hatchlings surviving to 15 days, or clutch size, respectively, with maternal age has been observed in *L. stagnalis* (Nakadera *et al.* 2014), *P. acuta* (Auld *et al.* 2014) and *A. arbustorum* (Baur 1990). However, as we are dealing with field-collected egg clutches, it is impossible to evaluate which hypothesis is correct in the population studied here.

The relationship between levels of multiple paternity on the one hand and mating frequency or partner number on the other hand is not a simple one (Dunn & Lifjeld 1994; Griffith 2007; Uller & Olsson 2008; Anthes *et al.* 2016). Based on the clutch parentage data, we found that the maximum number of male mating partners can be as high as nine, and the number of female mating partners as high as five (because snails sired offspring with up to five different mothers). However, these estimates ignore possible repeated copulations with the same partner, and may underestimate the number of mating partners for at least three reasons. First, we only sampled few clutches per individual snail at best, and just 60 clutches in total, obviously a limiting factor in a population consisting of thousands of snails that frequently produce a dozen or more clutches (A. Bürkli unpublished data). Hence, the true number of both male and female mating partners may be considerably higher. Second, male mating partners that failed to fertilize eggs will be overlooked, a scenario all the more realistic as *R. balthica* can probably digest allosperm (shown in a related species: Koene *et al.* 2009). And third, our sampling design is insensitive to fathers that sired less than 5% of the embryos in a clutch. As a consequence, any inference about the strength of sexual selection in the study population must necessarily be rather indirect.

Nonetheless, if variation in multiple paternity among individuals is indeed assumed to reflect variation in mating success, then there would be ample opportunity for sexual selection in the study population (Arnold 1994; Anthes *et al.* 2010). When further assuming that the detection

probability of mating partners (discussed in Anthes *et al.* 2016) does not change over time, sexual selection may increase in strength during the breeding season, in parallel to the observed increase in multiple paternity. This in turn may potentially create interesting seasonal dynamics regarding, for instance, an individual's optimal resource allocation to male and female function, or the presence and frequency of alternative reproductive strategies in the population. We know for sure that the distribution of paternity was skewed in > 90% of clutches. Although unequal siring success may in part result from pre-copulatory processes such as male rank in the copulatory sequence (Pélissié *et al.* 2014), commonly it is taken as evidence for considerable scope for post-copulatory sexual selection. In addition, copulation is unilateral in *R. balthica*, and so male and female mating number can be optimized independently, including sex-role specialization in the extreme case. In a species such as this, pre-copulatory sexual selection is expected to be particularly strong (Anthes *et al.* 2010). Although it has been known for some time that sexual selection can and does operate in hermaphroditic organisms (Anthes *et al.* 2010; Péliissié *et al.* 2012), this potential has rarely been shown in a natural population. Based on the results found here, *R. balthica* appears to be a promising species for future studies of sexual selection.

The number of fathers that sired a clutch was associated neither with clutch size nor with the likelihood of embryos to develop normally. In *R. balthica*, a species devoid of nuptial gifts and parental care, polyandry could increase female fecundity by providing females with nutritious ejaculates (Arnqvist & Nilsson 2000) or with seminal fluid proteins that redirect resource allocation towards egg-laying (Ram & Wolfner 2007; but see also Koene *et al.* 2010 for a study that found an opposite effect). A positive correlation between clutch size and the number of siring fathers could also arise as a simple by-product of larger clutches “sampling” a snail's allosperm

reservoir more thoroughly than smaller clutches. By contrast, female fecundity could be diminished if polyandry resulted in physical damage, physiological harm, reduced foraging time, increased predation rates, or increased risk of contracting a disease (e.g., Nahrung & Clarke 2007; Hoffer *et al.* 2010; Lange *et al.* 2012; Toft & Albo 2015).

Unlike clutch sizes, embryonic development rates could potentially be affected by the laboratory setting in which they were measured. Even though environmental conditions such as exposure to predator cues can alter the timing of onset of developmental events in this species (Rundle *et al.* 2011), we believe that the lack of a relationship between multiple paternity and embryonic development may be genuine. First, embryonic development rates were generally very high. Second, both singly and multiply sired clutches experienced the same artificial environment. And third, most clutches were not collected immediately after they were laid but rather a few days later, and thus embryonic development usually was started under field conditions.

The absence of differences in embryonic performance between singly and multiply sired clutches indicates that genetic benefits associated with more diverse, compatible, complementary, competitive or intrinsically better sperm cells (Zeh & Zeh 1996; Jennions & Petrie 2000; Bernasconi *et al.* 2003; Simmons 2005) may only appear after embryos have hatched or not at all. In its natural habitat, *R. balthica* is frequently exposed to both parasites (Wullschleger & Jokela 1999; Wiehn *et al.* 2002) and predators (Brönmark *et al.* 2011; Ahlgren & Brönmark 2012), and thus selective pressures may be rather heterogeneous in space and time. Testing for genetic benefits of polyandry at later stages of life, ideally by following the fate of singly and multiply sired progenies in the field, would thus be very interesting, although logistically difficult for obvious reasons. Another mechanism by which polyandry could increase embryonic performance

is fertilization assurance (Zeh & Zeh 1996), but we believe this to be less important in our study population. First of all, *R. balthica* is self-compatible (Pfenninger *et al.* 2011; Haun *et al.* 2012), although snails in the population studied here make use of this potential very rarely (Bürkli *et al.* 2017). Second, the frequency of unfertilized eggs was near-zero throughout the reproductive season (0.009% overall).

Purely correlative studies in open field populations such as ours face several difficulties when trying to measure fitness effects of polyandry. One potential risk is confounding number of mates with number of matings (Simmons 2005; Anthes *et al.* 2016). As stated earlier, we do not know how often snails in the study population copulated with the same partner repeatedly, how much the tendency to do so varied among individuals, nor how the effect of repeated copulations with one partner compares to that of polyandry. Observed effects of polyandry could also be biased by differences in maternal condition (Simmons 2005; Anthes *et al.* 2016). Finally, costs or benefits of polyandry could become visible only at the total female reproductive output, for example by influencing the rate at which clutches are produced, or they may manifest with respect to offspring size (see Sprenger *et al.* 2008a; Sprenger *et al.* 2008b). Investigating these possibilities requires further research. Polyandry-mediated effects on offspring number may also be rare in general, as brood size and genetically deduced levels of multiple paternity within broods were not correlated in a meta-analysis of 29 invertebrate species (Avise *et al.* 2011).

In conclusion, we showed that a natural population of the annual, simultaneously hermaphroditic snail *R. balthica* is characterized by substantial rates of multiple paternity. Coupled with skewed paternity shares in most egg clutches, this suggests that sexual selection may potentially be an important evolutionary force in the studied population. Both the frequency and magnitude of

570 multiple paternity strongly increased towards the end of the reproductive season, most likely as a  
571 result of sperm storage. Forces other than increased per-clutch fecundity and improved  
572 embryonic development must be driving the evolution of polyandry, for example fitness  
573 advantages of multiply sired progenies in heterogeneous environments later in life. Elucidating  
574 the selective pressures behind the evolution of polyandry, but also its proximate causes, will be  
575 the aim of future research.

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821 **Data Accessibility**

822 Microsatellite markers: GenBank accessions KX830983-KX830992

823 Microsatellite genotype and phenotypic data: Dryad DOI: <http://dx.doi.org/10.5061/dryad.2sk82>

824 and <http://dx.doi.org/10.5061/dryad.cq814>.

## 825 **Author Contributions**

826 A.B. and J.J. designed the project, analyzed the data and wrote the manuscript. A.B. organized  
827 sample collection and obtained all phenotypic and genetic data.

## Tables

**Table 1**

Sample sizes of collected and genotyped adult snails, egg clutches and embryos. Sampling was conducted in 2014 in a single natural population. We genotyped the embryos of seven clutches (five from sampling date 11.04.2014 and two from date 23.05.2014, respectively) as comprehensively as possible to assess the likelihood that all the fathers of a clutch were found in a random subsample of 15 genotyped embryos. To increase comparability between sampling dates the number of genotyped embryos from these clutches has been limited to 15 in this table. When including all genotyped embryos from these seven clutches, 361 embryos were genotyped in total on date 11.04.2014, with  $24.1 \pm 12.7$  ( $53.4 \pm 31.3\%$ ) genotyped embryos on average. Analogously, on date 23.05.2014, 231 embryos were genotyped in total, with  $15.4 \pm 1.4$  ( $55.9 \pm 23.9\%$ ) genotyped embryos on average.

Sampling date (days numbered continuously)	Number of adult snails collected (and genotyped)	Number of egg clutches collected (and genotyped)	Number of embryos genotyped (total number of embryos within the 15 clutches)	Mean number of genotyped embryos per clutch $\pm$ SD	Mean proportion (%) of genotyped embryos per clutch $\pm$ SD
06.03. (1)	85 (49)	26 (15)	223 (1023)	$14.9 \pm 1.4$	$28.3 \pm 21.2$
15.03. (10)	117 (0)	35 (0)	-	-	-
17.03. (12)	109 (0)	0	-	-	-
22.03. (17)	199 (58)	16 (15)	219 (746)	$14.6 \pm 1.3$	$38.3 \pm 20.5$
24.03. (19)	116 (0)	0	-	-	-
29.03. (24)	107 (0)	36 (0)	-	-	-
11.04. (37)	119 (51)	37 (15)	238 (770)	$15.9 \pm 1.4$	$34.9 \pm 13.6$
02.05. (58)	138 (0)	30 (0)	-	-	-
23.05. (79)	23 (23)	21 (15)	228 (499)	$15.2 \pm 1.3$	$54.9 \pm 22.4$

840 **Table 2**

841 Probability of detecting all the fathers of a clutch in a subsample of 15 embryos. Seven clutches were  
 842 genotyped as comprehensively as possible to estimate the proportion of subsamples that revealed the true  
 843 number of siring fathers (n = 1000 iterations). This proportion decreased with increasing rarity of the  
 844 rarest father (i.e. the father with the lowest number of sired embryos in a clutch).

Clutch	Sampling date	Number of eggs in clutch	Proportion (%) of genotyped embryos	True number of fathers (among all genotyped embryos)	Proportion (%) of subsamples revealing the true number of fathers	Proportion (%) of genotyped embryos sired by rarest father
04	11.04.	66	86.4	1	100.0	-
08	11.04.	36	100.0	3	42.5	2.8
18	11.04.	35	94.3	1	100.0	-
30	11.04.	35	91.4	2	100.0	40.6
31	11.04.	45	88.9	2	97.3	17.5
36	23.05.	20	85.0	3	89.3	5.9
46	23.05.	17	94.1	3	94.3	6.3

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846

847 **Table 3**

848 Assessing the quality of parentage assignments. COLONY (Jones & Wang 2010) provided for each  
849 embryo a list of potential full siblings  $1 \dots i$  along with their probabilities  $p_1 \dots p_i$  of being a full-sib of the  
850 focal embryo. The mean assignment probability of embryos to their full-sibs, computed by averaging  
851 these probabilities across embryos, represents a measure of the reliability of inferred full-sib families. Two  
852 additional indicators for the quality of parentage assignments are the mean inclusive and exclusive  
853 probability of inferred full-sib families. See Methods for details.

<b>Sampling date</b>	<b>Mean assignment probability of embryos to their full siblings <math>\pm</math> SD</b>	<b>Mean inclusive probability of full-sib families <math>\pm</math> SD</b>	<b>Mean exclusive probability of full-sib families <math>\pm</math> SD</b>
06.03.	$0.99 \pm 0.01$	$0.98 \pm 0.04$	$0.97 \pm 0.04$
22.03.	$0.99 \pm 0.02$	$0.96 \pm 0.05$	$0.96 \pm 0.05$
11.04.	$1.00 \pm 0.03$	$0.98 \pm 0.07$	$0.97 \pm 0.07$
23.05.	$0.99 \pm 0.03$	$0.99 \pm 0.06$	$0.95 \pm 0.13$

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855

**Table 4**

Seasonal variation in multiple paternity in a natural population. Levels of multiple paternity were estimated in field-collected egg clutches from four sampling dates in 2014 spanning the entire reproductive lifespan. The number of fathers per clutch was estimated by counting the number of embryonic alleles at the most diverse locus, which yields a minimum estimate, and from a parentage analysis using COLONY (Jones & Wang 2010) that infers the most likely number of fathers. Of multiply sired clutches with > 15 genotyped embryos, we identified the likely number of fathers present in exactly 15 randomly chosen embryos (n = 1000 iterations) to prevent measurement bias, but raw values are shown as well. The genotype of a parent was known in either one of two cases: if a clutch was sired by multiple fathers, and/or if in a singly sired clutch a parent was among the candidate parents. Parental roles can only be known in multiply sired clutches. For details see Methods and Supporting Methods.

Sampling date	Minimum number of fathers per clutch $\pm$ SD (allele counting)		Most likely number of fathers per clutch $\pm$ SD (COLONY)		Number of clutches with > 1 father (proportion)	Number of full-sib families	Number of unique parents (among candidate parents)	Number of unique parents with known genotypes (proportion)	Number of unique parents with known genotypes and parental roles (proportion)
	<i>raw</i>	<i>corrected</i>	<i>raw</i>	<i>corrected</i>					
06.03.	1.1 $\pm$ 0.4	1.1 $\pm$ 0.4	1.3 $\pm$ 0.6	1.3 $\pm$ 0.6	3 (20.0%)	19	32 (2)	11 (34.4%)	9 (28.1%)
22.03.	1.3 $\pm$ 0.5	1.3 $\pm$ 0.5	1.3 $\pm$ 0.5	1.3 $\pm$ 0.5	4 (26.7%)	16	27 (3)	11 (40.7%)	9 (33.3%)
11.04.	1.7 $\pm$ 0.7	1.6 $\pm$ 0.6	2.1 $\pm$ 1.4	2.1 $\pm$ 1.3	9 (60.0%)	32	43 (1)	35 (81.4%)	33 (76.7%)
23.05.	2.2 $\pm$ 0.7	2.2 $\pm$ 0.7	3.9 $\pm$ 1.9	3.8 $\pm$ 1.8	14 (93.3%)	56	48 (1)	46 (95.8%)	46 (95.8%)

**Table 5**

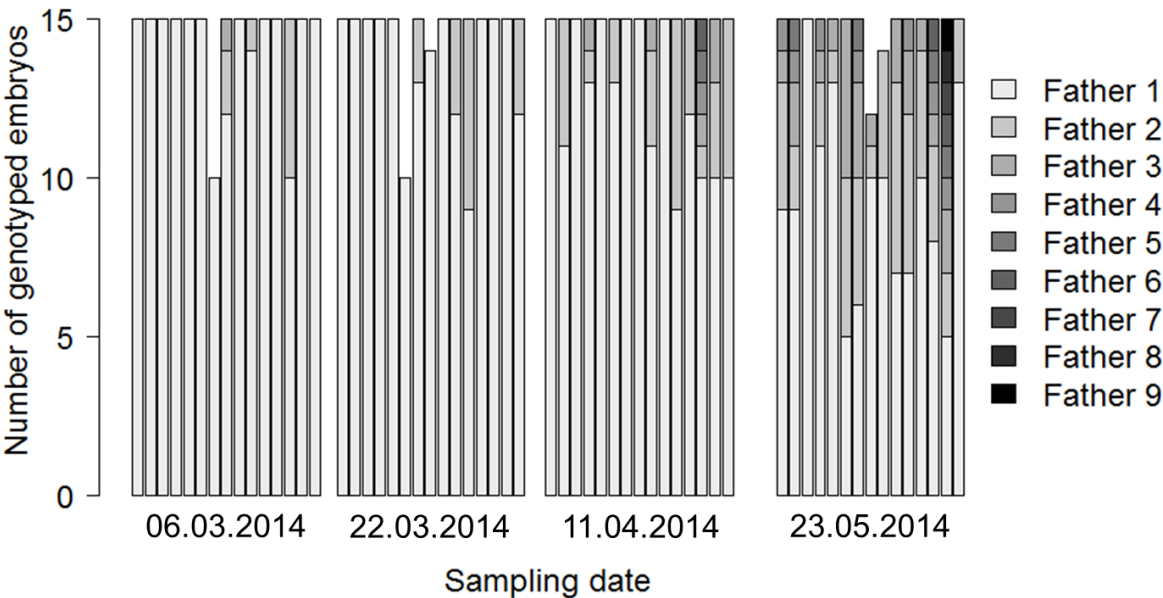
Number of “recaptured” parents. These are parents that contributed gametes to more than one genotyped egg clutch. The power to detect “recaptured” parents depends on the number of parents whose genotypes are known with certainty. As this number is not identical for all sampling dates (see Table 4), the number of “recaptured” parents should not be compared among sampling dates. Without exception, parents were “recaptured” in clutches collected on the same date. For details, see Supporting Methods.

<b>Sampling date</b>	<b>Number of fathers “recaptured” as fathers</b>	<b>Number of mothers “recaptured” as mothers</b>	<b>Number of parents “recaptured” in switched parental role</b>	<b>Proportion (%) of parents with known genotypes that were “recaptured”</b>
06.03.	1	0	0	9.1
22.03.	2	1	0 (1*)	27.3
11.04.	2	0	0	5.7
23.05.	12	1	3	34.8

\* On sampling date 22.03.2014 two clutches had exactly the same parents (2 fathers, 1 mother). In addition, the two fathers were the sole parents of a third clutch. Since this clutch was not multiply sired, parental roles could not be assigned, but by necessity this means that one of the two fathers “recaptured” as fathers was, in addition, also “recaptured” as a mother.

878 **Figures**

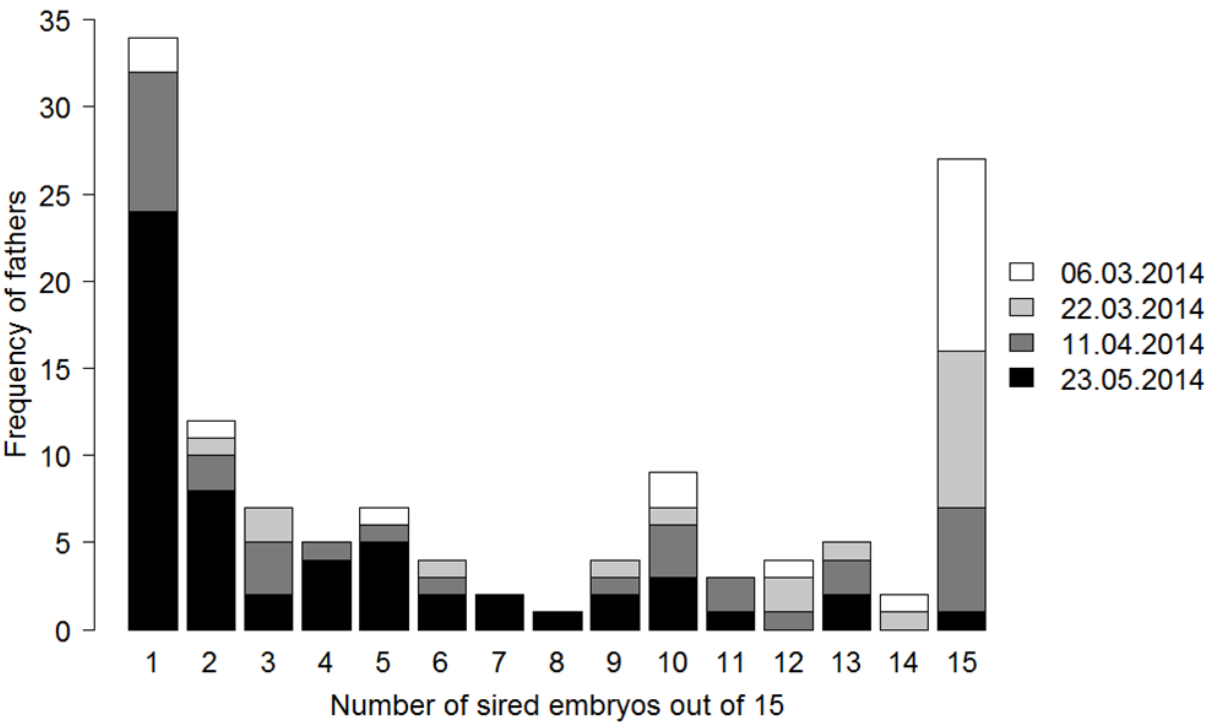
879 **Figure 1**



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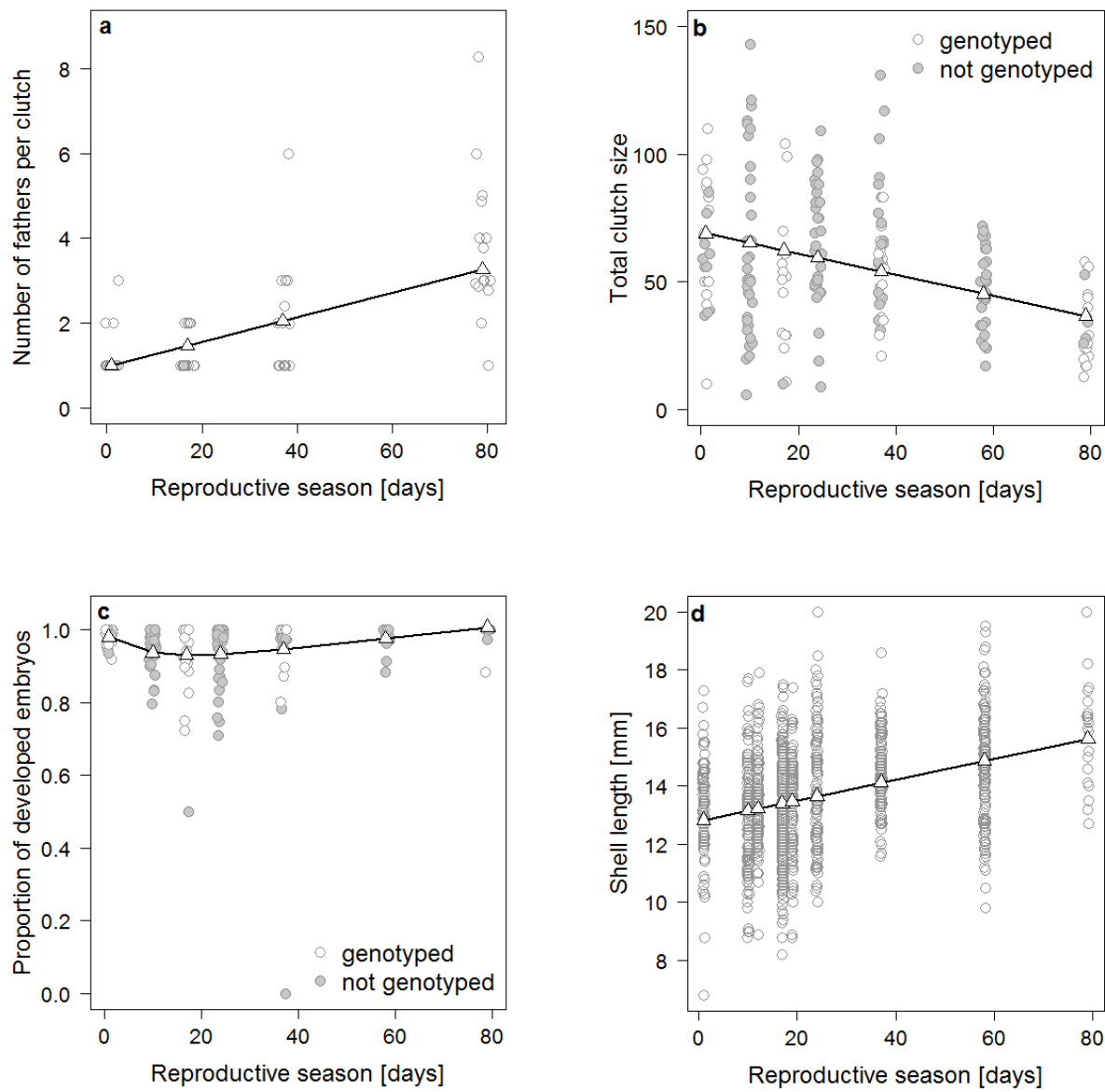
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882 **Figure 2**



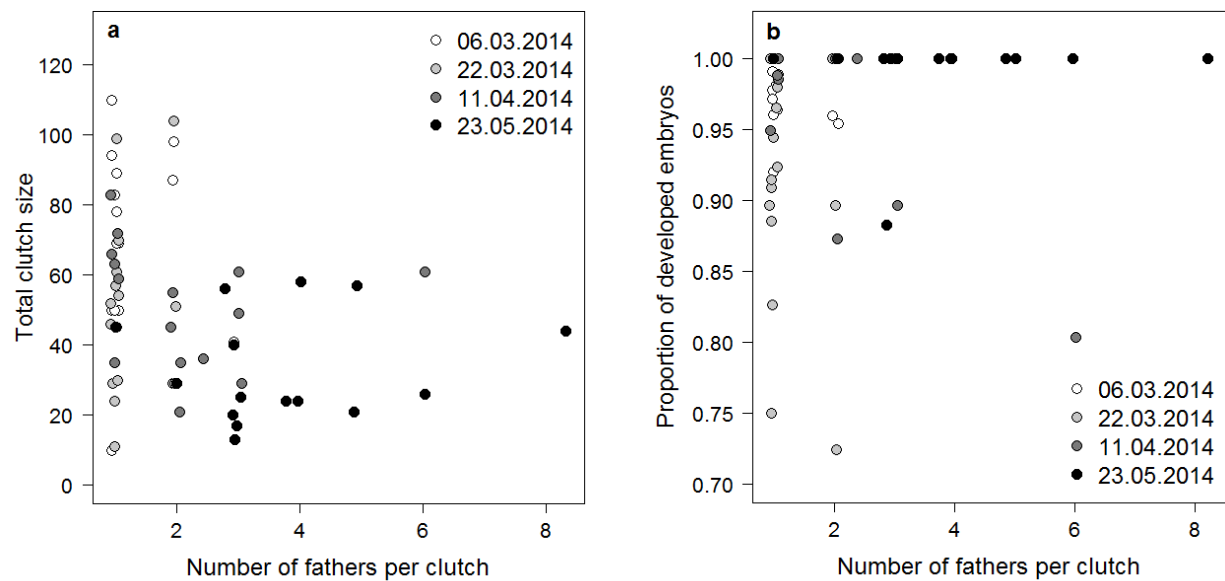
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884 **Figure 3**



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886 **Figure 4**



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## Figure legends

### Figure 1

Seasonal increase in multiple paternity in a natural population of *Radix balthica*. Shown are paternity distributions of 60 field-collected clutches from four sampling dates (15 clutches per date) spanning the entire breeding season, estimated using COLONY (Jones & Wang 2010). Each vertical bar depicts one clutch, with sections in different shades of gray corresponding to embryos sired by different fathers. The same shade of gray in different clutches does not indicate identical father genotypes. Spaces between temporal blocks are proportional to the number of days between sampling dates.

### Figure 2

Distribution of paternity shares across 60 field-collected egg clutches. Shown are the frequencies of fathers that sired exactly 1 embryo (rare fathers), 2-14 embryos, or 15 embryos (fully dominant fathers). Different shades of gray correspond to different sampling dates. The paternity shares shown here correspond exactly to the paternity distributions in individual clutches depicted in Figure 1 (for details see legend there and Methods).

### Figure 3

Seasonal changes in multiple paternity, clutch size, embryonic development, and adult shell length. (a) The most likely number of fathers that sired a clutch tripled over the course of the reproductive season ( $n = 60$  clutches). (b) Simultaneously, the average number of eggs per clutch decreased by half, while (c) the proportion of normally developed embryos per clutch first decreased slightly and then increased again ( $n = 201$  clutches). (d) Meanwhile, somatic growth in adult snails continued until they died at the end of the reproductive season ( $n = 1013$  adult snails). White triangles connected by line segments show model predictions (see Methods for details). The temporal change in the proportion of developed embryos per clutch was best approximated using the equation  $y = 1.56 - 0.38 * \text{sampling date} + 0.06 * (\text{sampling date})^2$  (adjusted  $R^2 = 0.06$ ,  $F_{2, 198} = 7.41$ ,  $p = 0.0008$ , linear effect of sampling date:  $t = -3.50$ ,  $p = 0.0006$ , quadratic effect of sampling date:  $t = 3.64$ ,  $p = 0.0004$ ).

### Figure 4

No relationship between levels of multiple paternity and clutch size or embryonic development. Different shades of gray correspond to different sampling dates. When accounting for seasonal changes in clutch size and in the proportion of developed embryos, the number of fathers per clutch did not have a significant effect.