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# Reproductive isolation keeps hybridizing *Daphnia* species distinct

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*Abstract*

We asked whether pre- (e.g., assortative mating, temporal isolation) or postzygotic (e.g., hybrid inviability, infertility) barriers are more likely to affect the hybridization between *Daphnia galeata* and *Daphnia hyalina*. We compared the taxonomic composition of different reproductive stages in the life cycle of *D. galeata*, *D. hyalina*, and their hybrids in Greifensee (Switzerland) by using molecular genetic methods. We found evidence for reproductive isolation between taxa and that hybrids in particular, have reduced sexual fitness. The results provide one potential mechanism for parental taxa to remain distinct. $F_1$ hybrid dominance in Greifensee could be explained by an increased asexual reproduction of hybrids that results in a higher proportion of gravid females compared with the parental *D. galeata*. The low sexual fitness of hybrids limits the abilities of hybrids to take advantage of diapausing eggs. The lower dispersal ability, including colonization of new habitats, and survival probability during harsh environmental conditions, could, therefore, lead to underestimates of historical hybrid occurrence by using diapausing egg bank reconstructions.

Hybridization and introgression are sources of variation that can influence adaptation to new environments and influence speciation in both plant and animal systems (e.g., Anderson and Stebbins 1954). Hybridization is a common phenomenon in both aquatic and terrestrial habitats (reviewed in Dowling and Secor 1997), but seems to be more common among plant species (25% species hybridize) than among animal species (10%, Mallet 2005). It occurs after secondary contact between partially reproductively isolated species and has several potential outcomes. Hybridization can lead to extinction of one or both species (reviewed in Rhymer and Simberloff 1996) to coexistence of parental species and hybrids (Moore 1977) to reinforcement (reviewed in Butlin 1987) or to merging into novel populations of reticulate or polyphyletic origin because of introgressive hybridization (reviewed in Arnold 1992).

The evolutionary significance of hybridization depends strongly on the frequency of hybridization events, as well as on hybrid fitness (reviewed in Arnold 1992). Both factors hinge on reproductive isolation mechanisms that can be found in various numbers, combinations, and strengths between the species involved (e.g., Arnold 1997), and be either pre- or postzygotic (reviewed in Avise 1994). Prezygotic isolation mechanisms include cytonuclear incompatibility and a range of behavioral and mechanical mating barriers (e.g., viability selection on migrants, assortative mating, egg-sperm recognition, and timing of reproduction). Postzygotic incompatibilities, on the other hand, comprise reduced hybrid fitness (e.g., offspring viability, fertility) as a consequence of genomic divergence and thus result in incompatibilities. Interactions between parental genomes can lead to a variety of positive and negative epistatic effects on the nuclear and cytonuclear level and result in a set of different genotypes of varying fitness (Burke et al. 1998). However, the final success of hybrids in a specific habitat depends on a complex genotype-by-environment interaction (e.g., Campbell and Waser 2001), as well as on stochastic effects related to extinction and colonization (Babik et al. 2003). Although, in general, hybrids seem to perform poorly compared with their parental species (Arnold 1997), under specific environmental conditions, they can be equally fit or more fit than their parents (Burke et al. 1998), which, in turn, can result in hybrid dominance (Milne et al. 2003).

Hybridization might be especially important among plant and animal species that can propagate both sexually and asexually. Even if $F_1$ hybrid genotypes have reduced sexual fertility, they may be able to perpetuate over years via asexual reproduction and reach high abundances. This should then increase the chance for successful sexual reproduction. In this way, asexual reproduction can facilitate the production of later hybrid generations (e.g., $F_2$ hybrids and backcrosses), self-fertilization, and finally, introgression into the parental population. For example, these mechanisms have been demonstrated in hybrid *Iris* populations (Burke et al. 2000).

Hybridization is especially common among zooplankton species, even across different genera of rotifers and cladocerans (reviewed in Hebert 1985), which reproduce by cyclical parthenogenesis. In cyclic parthenogens, long asexual phases are interrupted by short periods of environmentally induced sexual reproduction. In cladocerans, for example, females switch to the production of males

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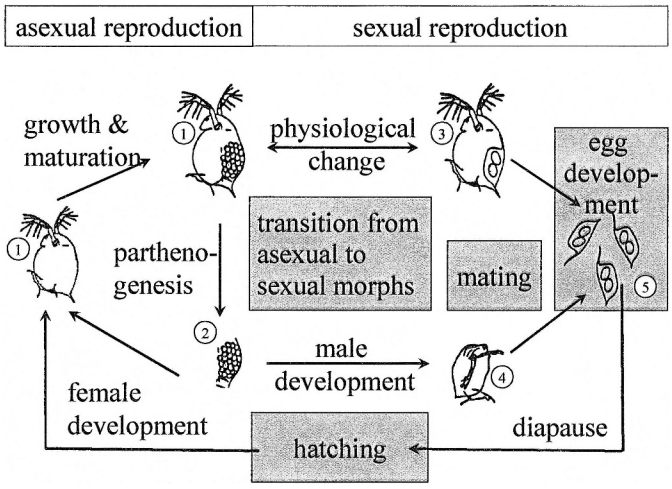


Fig. 1. Reproductive cycle of *Daphnia*, with critical steps (gray boxes) where reproductive barriers might occur. Numbers refer to the different reproductive stages: (1) asexual female, (2) asexual/male brood, (3) sexual female, (4) male, and (5) ephippia with ephippial eggs. (*Daphnia* drawings from De Meester 1990).

and sexual haploid ephippial eggs. Fertilized eggs are released in a protective structure called an ephippium and hatch after a period of diapause when exposed to hatching stimuli. Ephippia are important for colonizing new habitats and reestablishing local populations after unfavorable conditions. They can stay viable for decades (e.g., Carvalho and Wolf 1989).

The most frequently studied cyclic parthenogens in aquatic systems are cladocerans of the genus *Daphnia*. Hybrids between *Daphnia galeata*, *Daphnia cucullata*, and *Daphnia hyalina* are commonly found sympatrically with their parental species (*D. galeata-hyalina-cucullata* species complex) in permanent lakes across Europe. This co-occurrence of taxa can be explained by taxon-specific fitness differences indicated by distinct niche breadths (Weider 1993). Accordingly, fluctuations in biotic and abiotic conditions modify taxon-specific selection pressures and alter taxonomic composition. However, hybrids often combine life-history traits of their parents in a beneficial way (e.g., Schwenk and Spaak 1995), leading to $F_1$ hybrid dominance (e.g., Schwenk and Spaak 1995; Keller and Spaak 2004), also known as "temporal hybrid superiority" (proposed by Spaak and Hoekstra 1995). Long-term co-existence of parental species and hybrids (e.g., Keller and Spaak 2004) is only possible under conditions that also favor parental taxa and strengthen their competitive ability. For example, the parental taxon can be more resistant to a harmful parasite than its $F_1$ hybrid offspring (Wolinska et al. 2004). High hybrid competitive abilities have previously been discussed (e.g., Spaak and Hoekstra 1995), but the question remains why taxa do not merge. One possible explanation is the presence of reproductive isolation mechanisms that prevent parental species and hybrids from merging. Although observed differences in taxonomic composition between hatchlings from ephippial (sexual) eggs and active (asexual) lake populations (e.g., Keller and Spaak 2004) suggest the existence of reproductive isolation, these studies did not identify at which stage of the sexual *Daphnia* life cycle this bias occurs.

There are many stages within the sexual *Daphnia* reproduction cycle that could potentially be influenced by reproductive isolation mechanisms and lead to a shift in taxonomic composition between generations. These stages are (Fig. 1): "transition from asexual to sexual morphs" (induction of males and females, which produce gametes), "mating" (temporal and spatial co-occurrence of males and sexual females and their mating success), "ephippial (sexual) egg development," and "hatching." With "transition from asexual to sexual morphs," significant taxon shifts have been reported in the production of both sexual females and males (e.g., Spaak et al. 2004). Seasonal differences in the timing of the production of males and sexual females ("mating") were found among parental *Daphnia* taxa: *D. galeata* usually reproduce sexually in spring (e.g., Jankowski 2002), whereas *D. hyalina* and *D. cucullata* do so in fall (e.g., Spaak 1995). In some populations, however, sexual individuals of different parental taxa co-occur, enabling hybridization (e.g., Spaak et al. 2004). In addition to temporal separation, *Daphnia* taxa (e.g., Stich and Lampert 1981) or even sexes within the same taxon (Brewer 1998) can have different depth preferences in the water column. In the *D. galeata-hyalina-cucullata* species complex, however, no taxon- or sex-specific depth distribution has been found (Spaak et al. 2004). In a crossing experiment between two *D. galeata* and two *D. cucullata* clones, the presence of mating barriers was manifested in reduced mating success and low hatching rates of interspecific crosses (i.e., "ephippial egg development" and "hatching"; Schwenk et al. 2001). To date, however, the entire sexual reproductive cycle has not been studied for any natural hybridizing *Daphnia* population.

The aim of our study was to analyze the reproductive cycle in the *D. galeata-hyalina-cucullata* species complex and to examine at which stage reproductive isolation mechanisms arise. Life history traits explaining hybrid fitness and maintenance in asexual lake populations have been described in detail (e.g., Schwenk and Spaak 1995), but the processes controlling the genetic structure of hybridizing populations (e.g., the relative strengths of pre- and postzygotic barriers) are not well understood. Hence, we focused on the question of whether pre- or postzygotic barriers affect potential hybridization (Fig. 1; Table 1). Further, we tested the hypothesis that hybrids have reduced sexual fitness compared with parental taxa, which are traded off by increased asexual reproduction.

## Material and methods

*Study site and field sampling*—Greifensee (Switzerland) is a eutrophic, prealpine lake of medium size (8.5 $km^2$ surface area; 33 m maximum depth) that has undergone a sharp decline in total phosphorus concentration for the last 25 yr (Keller et al. 2002). Greifensee is inhabited by *D. galeata*, *D hyalina* and their interspecific hybrids. During spring and fall, in which sexual reproduction usually occurs (see Keller and Spaak 2004), the *Daphnia* population was screened

Table 1. Summary of the aspects of the sexual life cycle of hybridizing *Daphnia* analyzed to detect the presence and strength of pre- and postmating reproductive isolation mechanisms.

| Analyzed stages | Questions | Response variables | Predictions |
|---|---|---|---|
| Transition from asexual to sexual morphs | Do parental taxa produce relatively more sexual females? | Frequency of pure parental taxa in asexual and sexual females (spring 2000, 2002, 2004, and 2005). (including data from Keller and Spaak 2004). | In absence of reproductive isolation the asexual population should participate randomly in sexual reproduction and therefore clonal and taxonomic composition should be the same between asexual and sexual stages |
| | Do parental taxa produce relatively more males? | Frequency of pure parental taxa in asexual females and males (fall 2003, 2004, and spring 2004, 2005). | |
| | Do all clones invest equally in sexual reproduction? | Clonal diversity in asexual females and sexual stages (males and sexual females). | |
| Mating | Do sexual females and males co-occur spatially? | Vertical depth distribution of males and sexual females at noon and midnight. | Both sexes should show overlapping depth distribution to enable mating. |
| Egg development | Are ephippia of hybrids more often empty than these of parental species? | Number of pure parental and hybrid sexual females with and without ephippial eggs (pooled data from 32 checked females sampled in spring 2004 and 59 in 2005, respectively). | In absence of reproductive isolation hybrids and parental taxa should have equal proportion of empty ephippia. |
| | Are ephippial eggs produced by random mating? | Number of pure parental and hybrid ephippial eggs (spring 2004, spring 2005); Expected hybrid-parental distribution—calculated from maternal and paternal taxa composition (see text) | Observed taxonomic composition should correspond to the one calculated under the assumption of random mating. |
| Hatching | Does the hybrid-parental taxon distribution of matured hatchlings correspond with that in ephippial eggs? | Number of pure parental and hybrids among ephippial eggs and hatchlings (spring 2004). | No difference between taxonomic composition of ephippial eggs and hatchlings would be expected in the absence of isolation mechanisms. |
| | The first step in hybridization, i.e., the $F_1$ hybrid production, as shown in other species, is it also in *Daphnia* the most difficult to obtain? | Proportion of parental and hybrid ($F_1$ hybrids and backcrossed) hatchlings from floating ephippia (4-yr observation period; including data from (Keller and Spaak 2004). | $F_1$ hybrids, backcrosses and parental taxa should occur randomly in matured ex-ephippial hatchlings. |

weekly for the presence of sexual stages (in the years 2004 and 2005). Zooplankton samples were collected with a 250-$\mu$m net hauled through the entire water column at three locations around the deepest part of the lake. Samples were cooled with ice during transport to the laboratory and then were analyzed. The body size of 70–100 randomly chosen asexual females was measured from the top of the eye to the base of the tail spine, and clutch presence was determined. Adult asexual females, and sexual females and males were collected for genetic analysis. In spring 2004, ephippia that were floating on the water surface were sampled by carefully skimming wide areas of the water surface with a 250-$\mu$m plankton net. These ephippia were stored for several months and later exposed to hatching stimuli (i.e., long-day photoperiod with 16 h light and 8 h dark at 12°C; for a detailed sample description and hatching method see Keller and Spaak 2004).

Lastly, the overlap in range of males and sexual females in the water column was investigated. For this, we used a closing net to sample quantitative 5 m (30–10 m) and 2.5 m (10–0 m) depth intervals at midday and midnight on two days (28 April and 02 May 2005).

*Molecular markers*—Two sets of genetic markers were used for taxa identification. First, cellulose acetate allozyme electrophoresis was used to analyze four polymorphic enzyme loci (PGI, PGM, AAT, AO, after Keller and Spaak 2004). AO and AAT are diagnostic markers for *D. galeata*, *D. hyalina*, and *D. cucullata* (Wolf and Mort 1986; Gießler 1997). Second, to distinguish diagnostic haplotypes in the *D. galeata-hyalina-cucullata* species complex, restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer region (ITS) gene region (ITS2, 5.8S, ITS1 and part of 18S) were performed (hereafter referred as ITS-RFLP). The ITS gene region was amplified by following the PCR based procedure of Billiones et al. (2004) and was cut with two restriction enzymes, *MWO* I (NEB; 60°C) and *SAU*96 (NEB; 37°C). Visualization of

fragments was performed on a 2% agarose gel (110–115 volts).

*Sampling procedure and deoxyribonucleic acid extraction*—All individuals were analyzed with allozyme electrophoresis. All sexual females and males (except males in 2004) and a random subsample (20 individuals per sample) of asexual females, were further classified with ITS-RFLP. For the combined allozyme and RFLP analysis, the following procedure was used: 12 well sample plates (Helena Laboratories) were sterilized for 30 min with bleaching agent (Eau de Javel) and subsequently exposed to ultraviolet radiation (>30 min). Deoxyribonucleic acid (DNA) contamination of the plates was tested by running a polymerase chain reaction (PCR) with only autoclaved, ultrapure water as a template. Individual *Daphnia* were placed in each well of the plates with 4 $\mu$L autoclaved, ultrapure water and were homogenized with a spatula. The spatula was sterilized between every homogenization step with a bleaching agent. For DNA preparation, 1 $\mu$L of each homogenate was incubated for 12 h in 25 $\mu$L H3 buffer (10 mmol $L^{-1}$ Tris-HCl; pH 8.3 at 25°C, 0.05 mol $L^{-1}$ potassium chloride, 0.005% Tween-20 and 0.005% NP-40) and 1.0 $\mu$L Proteinase K (Roche, 18.2 mg $mL^{-1}$). Proteinase K was deactivated by heating the sample for 12 min to 95°C. PCR and the digesting procedure for ITS-RFLP was performed as described before. For allozyme electrophoresis, 1 $\mu$L of ultrapure water was added to the remaining *Daphnia* homogenate and after the first two applications for AO and AAT, 2 $\mu$L was added, to ensure enough homogenate volume for PGI and PGM (for a more detailed description see Keller and Spaak 2004).

Ephippial eggs were analyzed separately from their mothers. Because of the limited amount of cell material, they could only be analyzed for ITS-RFLP. Sexual females were placed on a microscope slide with some drops of autoclaved ultrapure water, and ephippia were removed. Ephippia were then placed on a new microscope slide with 100 $\mu$L of H3 buffer. The ephippial case was removed and eggs separated from each other. Each egg was squashed, and its content was transferred to 30 $\mu$L H3 buffer for subsequent DNA isolation and ITS-RFLP analysis.

*Identification of hybrid classes*—Pure *D. galeata* and $F_1$ hybrids ($F_1$ hybrids between *D. galeata* and *D. hyalina*) were identified with the NewHybrid software (Anderson and Thompson 2002), by using four polymorphic allozyme loci (PGI, PGM, AO, AAT). The identification probability was $\geq 95\%$. The remaining individuals (probability, <95%) were pooled and labeled as ''backcrosses.'' Furthermore, loci were used to distinguish between different multilocus genotypes (MLG). Clonal diversity $D$ was calculated as the negative logarithm of Simpson's index of concentration $C$ (see Pielou 1975) and is a composite of abundance and evenness (low values indicating a dominance of a single MLG; high values indicating a high number of equally abundant MLGs).

Taxon classification using the ITS-RFLP marker was based on the dichotomous key of Petrusek et al. (2005) and was used for direct comparisons among ephippial eggs, asexual females, sexual females, males, and hatchlings (except for males in 2004, where only allozyme markers were available). With this method, however, some *D. galeata* and *D. galeata* hybrids are incorrectly classified as *D. cucullata* and *D. cucullata* hybrids because of a mutation at the restriction site of *MWO* I (Skage et al. in press). Individuals with no *D. cucullata* alleles in the two diagnostic allozyme markers (AO, AAT), but with *MWO* I *D. cucullata* band pattern, were considered as misclassified. Therefore, homozygous individuals that had a *D. cucullata* banding pattern and heterozygous individuals that had a *D. galeata* × *cucullata* banding pattern were classified as *D. galeata*, and individuals that had a *D. cucullata* × *hyalina* banding pattern were classified as *D. galeata* × *hyalina* hybrids (Skage et al. in press). Analyses with three diagnostic markers (AO, AAT, and ITS-RFLP) enabled a more precise hybrid classification. Individuals were classified as ''pure parental'' if they contained exclusively alleles of one taxon at all loci, or otherwise, as hybrids. For the taxonomic classification of ephippial eggs, we used ITS-RFLP and our knowledge of their maternal genotypes. An ephippial egg was only classified as ''pure parental'' if it was ''pure'' based on ITS-RFLP and if its mother was also classified as a ''pure parental'' taxon. All other eggs were pooled into the hybrid category.

*Analysis of sexual reproductive cycle in hybridizing* Daphnia *and statistical analysis*—The reproductive cycle of *Daphnia* is divided into the parthenogenetic (clonal) and sexual cycle. In the sexual cycle, selective processes can cause deviations from random mating at several stages (Fig. 1). The different aspects that were analyzed to specify occurrence and strength of pre- and postmating isolation mechanisms are summarized in Table 1. $R \times C$ test of independence (Sokal and Rohlf 1995) was used to test for differences in numbers of parental and hybrid sexual females with and without eggs. Further, this test was used to check for taxa differences between numbers of specific reproductive stages at the ''egg development'' stage (i.e., asexual females, sexual females, males, expected offspring, ephippial eggs, and hatchlings; Sokal and Rohlf 1995). At the ''mating'' stage, we performed a Kolmogorov-Smirnov two-sample test to detect differences in clonal diversity between asexual and sexual stages. Furthermore, analysis of variance (ANOVA) with reproductive stage, reproductive season (spring or fall), and sampling date (nested in reproductive season) as main effects, was used for taxonomic comparison of asexual females and sexual females. For the ANOVA, only those reproductive seasons with at least two samples per season were included. Because of a limited number of seasons with at least two successive samples, we incorporated the single sample from fall 2003 in the analysis and performed a one-tailed paired $t$-test to compare males and asexual females. Frequency data were arc-sin square-root transformed (Sokal and Rohlf 1995). Assumptions for the calculation of expected taxonomic composition of ephippial eggs were random mating, exclusion of multiple mating events, and that pure offspring can only result from pure parental taxon crosses. Finally, we tested (by analyzing proportions of gravid females) if $F_1$

hybrids have an increased asexual reproduction compared with the parental taxon *D. galeata*, which might explain their dominance in the asexual population. The binomial trait ("gravid" or "nongravid") was analyzed with a generalized linear model, by assuming a binomial error distribution and a logit link function. For this test, we considered the sample dates from the long-term population survey (1998–2005; see also Keller and Spaak 2004) with at least 5 mature females (≥1 mm; J. Wolinska unpubl. data) per group. All analyses were performed by using STATISTICA for Windows, release 7.1 (StatSoft).

## Results

The parental taxa invested significantly more than hybrids in the production of sexual females (reproductive stage: $F_{1,6} = 20.73$, $p = 0.004$); but for male production the tendency was opposite although not significant (*t*-test: $t = -1.71$, df = 8, $p = 0.063$) (Fig. 2). The relative frequency of *D. galeata* sexual females was variable over the study period, resulting in a significant season effect ($F_{6,3} = 14.123$, $p = 0.004$), as well as a significant reproductive stage × season interaction ($F_{6,3} = 9.93$, $p = 0.01$). Individual clones (MLG-genotypes) also varied in their participation in sexual reproduction. In particular, one clone was almost absent among sexual females (max. 2.1% in April 2004), whereas it was the dominant clone in males during fall 2003 (89.5%) and spring-summer 2004 (78.8%; Fig. 3). The presence of this dominant "male-MLG," however, did not alter the outcome of the analyses in males (*t*-test, excluding dominant "male-MLG": $t = -0.49$, df = 8, $p = 0.319$). The clonal diversity in males was significantly lower compared with asexual females (Kolmogorov-Smirnov test: $p < 0.005$). Clonal diversity of asexual females was comparable with that of sexual females (Kolmogorov-Smirnov test: $p > 0.1$) (Fig. 4).

At the "mating" stage, we found that the depth distributions of males and sexual females overlapped during both day and night. At the "egg development" stage, 50.0% of the checked ephippia were empty in 2004, and 32.2% in 2005, respectively. We found more empty ephippia produced by hybrid females than by pure parental types ($G = 4.134$, df = *1*, $p = 0.042$). We also detected significantly fewer hybrid genotypes in the ephippial eggs than would be expected if mating was random (2004: $G = 6.623$, df = *1*, $p = 0.010$; 2005: $G = 52.426$, df = 1, $p < 0.001$; Fig. 5). At the "hatching" stage, we found that fewer hybrids hatched than would be expected from the genotype distribution of ephippial eggs (2004: $G = 4.606$, df = 1, $p = 0.032$). In the 490 hatchlings we analyzed from four consecutive years, only 1.2% were $F_1$ hybrids and 23.3% were *D. galeata* backcrosses, whereas all the rest (75.5%) were pure *D. galeata* (based on the NewHybrid software of Anderson and Thompson [2002]).

For the analysis of $F_1$ hybrids' asexual reproductive success, a total of 163 parental *D. galeata* and 423 $F_1$ hybrids collected on 17 different dates between 2002 and 2005 were analyzed. We found significantly more gravid females in $F_1$ hybrids (72%) than in *D. galeata* (46%) (Wald $\chi^2_1 = 33.633$, $p < 0.001$).

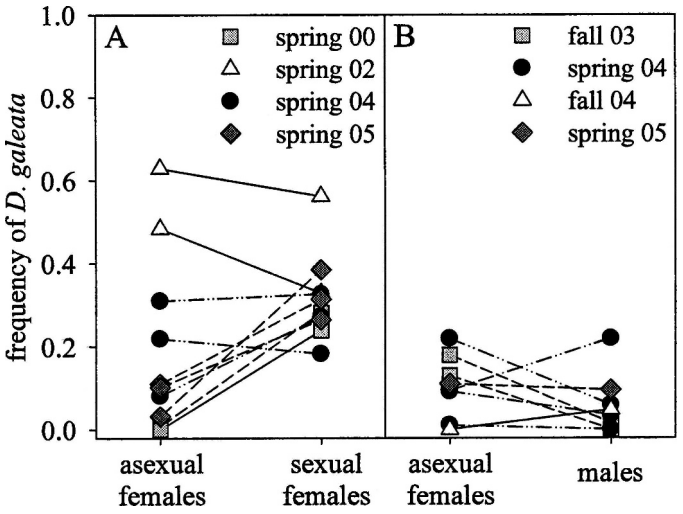


Fig. 2. Comparison of *D. galeata* frequency among asexual and sexual stages on different sampling dates and seasons: (A) asexual females and sexual females; (B) asexual females and males.

## Discussion

We found clear evidence for reproductive isolation between taxa within the *D. galeata-hyalina-cucullata* species complex. Although *D. galeata* is much less abundant than the first generation of *D. hyalina* × *D. galeata* hybrids ($F_1$ hybrids) in the lake (Keller and Spaak 2004), *D. galeata* dominates the sexual reproduction phase. Reproductive isolation might be the reason why parental species within the *D. galeata-hyalina-cucullata* species complex are still morphologically and genetically distinguishable even though hybridization among all three taxa is widespread and common.

Previous studies found taxonomic shifts among asexual, sexual stages, and hatchlings in hybridizing *Daphnia* (Jankowski 2002; Keller and Spaak 2004). From these studies, it is not clear if the observed differences were the result of pre- or postzygotic mating barriers. Incorporation of kinship relations (i.e., direct comparison of mothers and daughter eggs) helps to disentangle the exact stage at which mating barriers occur. At the "transition from asexual to sexual morphs" stage, we found directionality toward *D. galeata* in sexual females, whereas, in male production, there was a tendency toward hybrids. One potential explanation for this difference could be in varying energy demands of sexual eggs and male production. Ephippial production consumes energy reserves accumulated over two preceding instars (Lynch et al. 1986), whereas the production of males seems to be energetically similar to asexual propagation, because male and parthenogenetic progeny can be produced in the same clutch (Olmstead and Leblanc 2002). Further, we found that sexual reproduction can, in addition, be biased at the clonal level, because a single genotype dominated the whole male group for two successive seasons (Fig. 3), resulting in an overall low clonal diversity within males (Fig. 4). This is in accordance

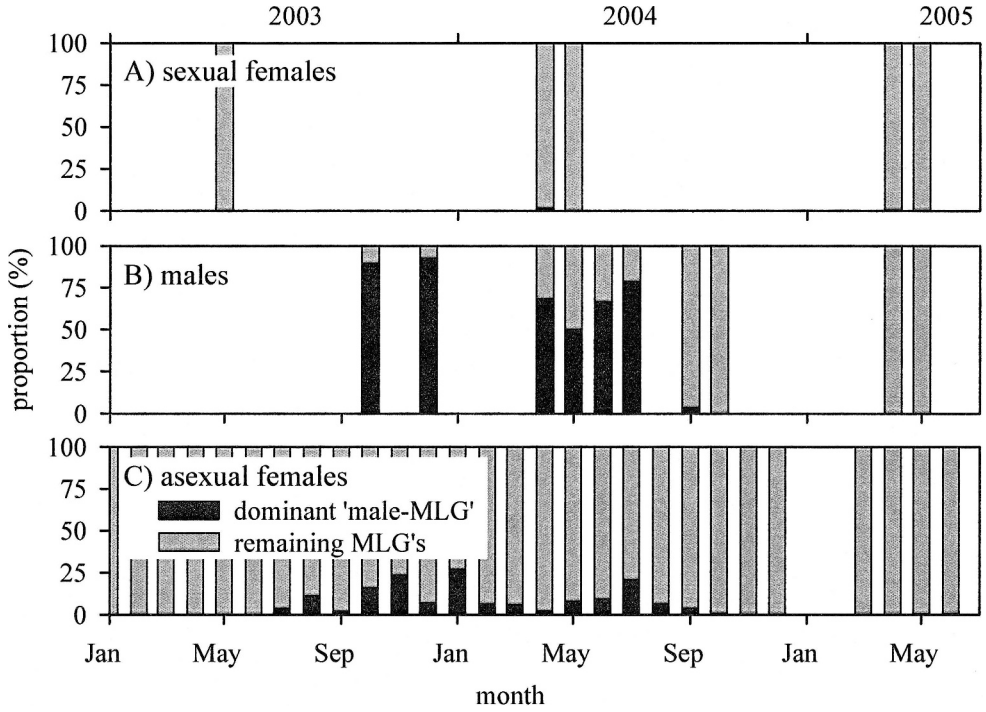


Fig. 3. Temporal fluctuations of the dominant male- MLG, (a "backcross" genotype) in (A) sexual females, (B) males, and (C) asexual females in comparison with all other MLGs (Greifensee; January 2003 to July 2005, samples pooled monthly; sexual stages: only data shown with >20 males or sexual females sampled).

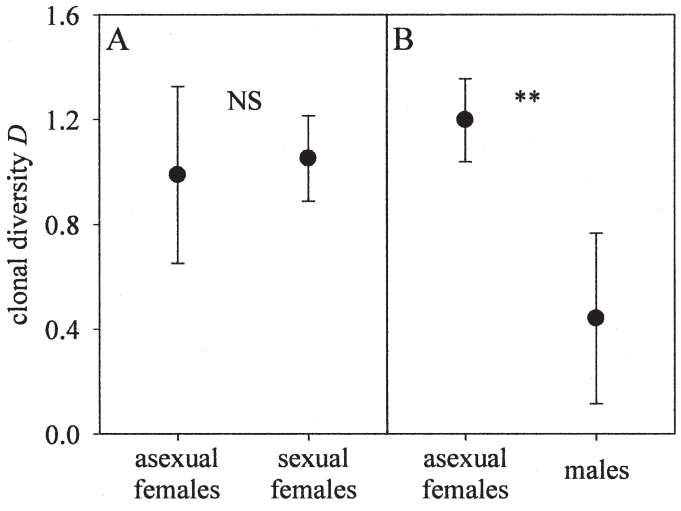


Fig. 4. Clonal diversity $D$, calculated as the negative logarithm of Simpson's index of concentration in asexual females and (A) sexual females (spring 2000, 2002, 2004, and 2005) and (B) males (fall 2003, 2004, and spring 2004, 2005). Mean values (± standard deviation) are shown. Diversity differences between asexual and sexual stages were tested with a Kolmogorov-Smirnov two sample test (**$p < 0.01$, NS = not significant).

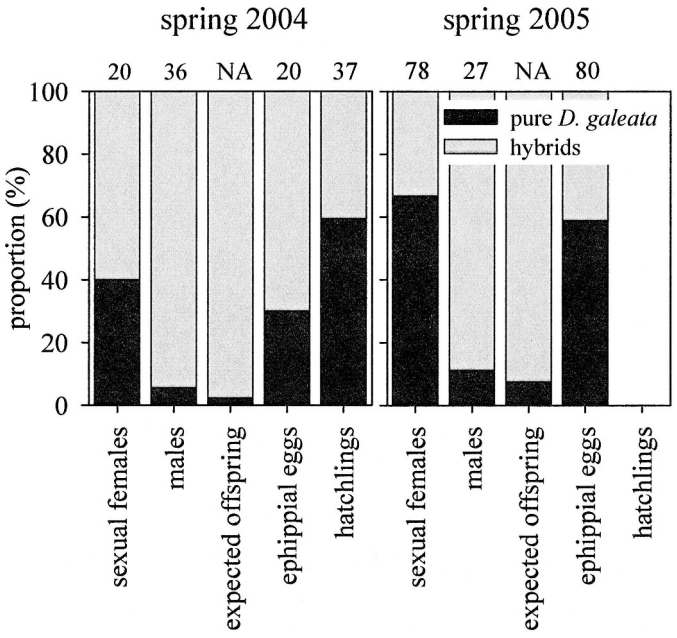


Fig. 5. Proportional hybrid-parental distribution of sexual females (mothers), males (potential fathers), expected offspring, ephippial eggs (observed offspring), and hatchlings in Greifensee. For the assumptions for the calculation of expected offspring see text. The top of each bar indicates the number of analyzed individuals, NA refers to calculated proportions.

with studies on *D. pulex* where clonal differences in contribution to sexual reproduction are common (Innes and Dunbrack 1993) and for that reason suggests a general phenomenon in *Daphnia*. However, clonality occurred only in males, not in sexual females and, therefore, it did not affect the observed pattern in the superimposed taxonomic participation in sexual reproduction.

The next stage in successful hybridization is ''mating,'' which requires temporal and spatial co-occurrence of males and sexual females. In the *D. galeata-hyalina-cucullata* species complex, hybridization is a regularly occurring process (e.g., Jankowski 2002) and taxon specific diel vertical migration of sexual stages has not been detected within this complex (e.g., Spaak et al. 2004). Accordingly, we found that most sexual females and males stay day and night in the uppermost 10 m of the water column thereby providing sufficient sex overlap to enable mating.

We detected many empty ephippia in the water column at the ''egg development'' stage, which were most likely released by sexual females when the eggs were not fertilized (see also Keller and Spaak 2004). Empty ephippia have also been found in experimental crosses between *D. galeata* and *D. cucullata* (Schwenk et al. 2001) and in intraspecific crosses (e.g., *D. magna*, Boersma et al. 2000). It seems that empty ephippia are a common phenomenon in sexual reproduction in *Daphnia*. We found a significant relation between the maternal taxon and the proportion of empty ephippia. Hybrid genotypes had a larger proportion of empty ephippia, and thus a reduced sexual fitness, compared with the parental taxon *D. galeata*. The further reduction of hybrid fitness was confirmed by our finding that there were fewer viable ephippial eggs with a hybrid genotype than expected by random mating (Fig. 5). This bias might be caused by problems with fertilization or egg development.

In general, reproductive isolation mechanisms among taxa should most strongly affect the initial hybridization stages because $F_1$ hybrids are the most difficult to produce (reviewed in Mallet 2005). We found low proportions of $F_1$ hybrid hatchlings in hybridizing *Daphnia*, compared with parental *D. galeata* hatchlings. Although backcrossed *D. galeata* occurred more often than $F_1$ hybrids, they are produced rarely. This overall low portion of ephippial hybrid sexual eggs limits the dispersal ability of hybrids, including colonization of new habitats and survival probability during harsh environmental conditions, and might lead to strong underestimation of historical hybrid occurrences based on diapausing egg bank analysis. Regarding to the low production rate of hybrids, it has been shown that hybrids can suffer from reduced sexual fitness (e.g., Johannesson et al. 1995), although this is not a general rule (e.g., Willi and Van Buskirk 2005). We suggest that the often-observed $F_1$ hybrid dominance of asexual *Daphnia* populations (e.g., Keller and Spaak 2004) is maintained by asexual reproduction, which masks sexual hybrid inferiority. $F_1$ hybrid superiority can be possible through an increased fitness of hybrids compared with parental species; for example, reduced mortality or enlarged fecundity (i.e., higher proportion of gravid females or larger clutch sizes). Indeed, we found higher proportion of gravid females among $F_1$ hybrids than among parental *D. galeata*. Clutch sizes, however, do not differ between the two taxa (J. Wolinska, unpublished data). Hence, the cyclic parthenogenetic reproduction strategy of *Daphnia* seems to promote $F_1$ hybrid success.

The ability to reproduce both asexually and sexually is particularly important in two common groups of freshwater zooplankton: cladocerans and monogonont rotifers (Hebert 1987), but it is also common in many plant species (e.g., Ellstrand et al. 1996). The frequency of asexual reproduction is often increased in hybrids, and thought to maintain hybrid populations (e.g., Ellstrand et al. 1996), even with very limited hybrid sexual fitness or sterility. Therefore, hybrids might be able to outperform their parents in both parental and hybrid habitats (Schwenk and Spaak 1995; Emms and Arnold 1997). Further, it has been proposed that asexual propagation can increase the mating probability in sparse populations (Gerritsen 1980). This means for *Daphnia* that a high frequency of a single clone may lead to an increased number of mating trials for this clone and therefore increase the chance for successful mating. Hence, the reduced sexual fitness of hybridizing species, as found in our study and in an *Iris* system (Johnston et al. 2004), might be compensated by high abundances of asexual hybrid clones (Emms and Arnold 1997). We found remarkable proportions of backcrossed hybrid hatchlings, suggesting that the low sexual fitness of $F_1$ hybrids in hybridizing *Daphnia* can be compensated generally through a high number of mating events. Asexual reproduction might, therefore, indirectly increase the probability that new unique hybrid genotypes will be created. Under specific conditions, some of these new genotypes are obviously fitter than either parental type, leading to the widespread $F_1$ hybrid dominance in hybridizing *Daphnia* taxa.

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