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Spatial and temporal patterns of sexual reproduction in a hybrid *Daphnia* species complex

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Evidence for extensive interspecific hybridization among species of the genus Daphnia has been accumulating on a global scale. Although there is evidence for limited gene flow between taxa via hybridization, many species still maintain discrete morphological and molecular characteristics. We studied temporal and spatial patterns of sexual reproduction within the Daphnia galeata–hyalina–cucullata hybrid species complex in a lake (Plußsee), located in northern Germany. Allozyme electrophoresis allowed us to track seasonal changes in taxon composition as well as the quantification of back-crosses. Sexually-reproducing animals (ephippial females and males) were mainly found in autumn. The simultaneous presence of sexual morphs of D. galeata and D. galeata × hyalina with the dominant D. hyalina taxa makes recent hybridization, as well as back-crossing, plausible. Males and ephippial females of D. hyalina were not back-crossed as were the parthenogenetic females. The low number of sexual clones of the hybrid D. galeata × hyalina might reflect its reduced fertility, although these few clones were detected in high densities. Only hybrid-clones that had a back-cross genotype (towards D. hyalina) exhibited ephippial females and males. This indicates that male and ephippial female production within the Daphnia taxa is not random, which might increase the chance for the parental Daphnia species to remain distinct.

INTRODUCTION

As a result of decreased fertility, interspecific hybridization among animals has long been considered to be an evolutionary dead-end for many taxa (Mayr, 1942; Barton and Gale, 1993). Recent studies (Arnold, 1997) on various animal species, e.g. birds (Grant and Grant, 1994, 1996), fish (Dowling and Demarais, 1993), and toads (Nurnberger *et al.*, 1995), have, however, shown that hybrids may play important evolutionary and ecological roles. Because gene combinations are formed that normally do not occur, hybridization is often viewed as a potentially important mechanism for creating many different genotypes in a short period of time, leading ultimately to speciation events (Arnold, 1997).

Of special interest in elucidating interspecific hybridization processes are studies of cyclically or obligately parthenogenetic animals, which are able to circumvent possible decreased hybrid fertility. In particular, interspecific hybridization among members of the freshwater cladoceran *Daphnia* (Crustacea, Anomopoda) has been studied extensively during the last decade (Gießler, 1997b; Schwenk and Spaak, 1997; Spaak, 1997; Reid *et al.*, 2000; Schwenk *et al.*, 2000). Hybridization within the *Daphnia galeata–hyalina–cucullata* hybrid species complex (*D. galeata* Sars, *D. cucullata* Sars, *D. hyalina* Leydig) is very common and hybrids of these *Daphnia* species are found in many European lakes. These hybrids often co-occur with one or both of their parental species and in many cases hybrids are reported to be the dominant taxon within these populations (Wolf, 1987; Spaak and Hoekstra, 1993, 1997). Although *Daphnia* hybrids also seem capable of reproducing sexually, parthenogenetic reproduction

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throughout most of the year enables *Daphnia* hybrids to increase their population density very rapidly and to maintain populations over a long period of time. Life-history experiments on hybrids and parental taxa have shown that some environmental conditions might actually favour hybrids thanks to their advantageous combination of parental traits (Weider, 1993; Boersma and Vijverberg, 1994; Spaak and Hoekstra, 1995, 1997) and therefore lead to temporary hybrid superiority (Spaak and Hoekstra, 1995; Spaak *et al.*, 2000).

In general, *Daphnia* reproduce parthenogenetically most of the year. Sexual phases are restricted to distinct periods when environmental conditions deteriorate (e.g. in autumn). Under stressful conditions, parthenogenetic *Daphnia* switch to sexual reproduction, which leads to the production of diapausing eggs. A number of factors that induce sexual reproduction in daphnids have been proposed, including temperature, photoperiod and population density (Carvalho and Hughes, 1983; Korpelainen, 1989; Hobæk and Larsson, 1990), as well as fish exudates (Slusarczyk, 1995). The induction of diapause in *Daphnia* seems to be maternally controlled (Alekseev and Lampert, 2001), indicating that the photoperiod and food levels experienced by the mothers are the main factors. During the sexual phase, diapausing eggs encased in a resistant structure (ephippium) are produced. Ephippia serve the dual function of both allowing for either an escape in time (via dormancy), or an escape in space (via dispersal), and serving as a reservoir of recombined genotypes on which natural selection (clonal selection) can operate (Spaak, 1995).

If two *Daphnia* taxa produce sexual forms simultaneously then interspecific mating, hybridization, might occur. Recent studies have shown that hybrids may be produced regularly (Taylor and Hebert, 1992; Müller and Seitz, 1994; Spaak, 1997) and as a result of the presence of fertile hybrids, back-crossing can take place (Spaak, 1996; Schwenk, 1997). However, the various *Daphnia* taxa are still found to be distinct, based on morphological characteristics and molecular markers (e.g. allozymes, random amplified polymorphic DNA), and both back-crossing and directional introgression seem to be limited (Schwenk and Spaak, 1997). This suggests the existence of pre- or post-mating barriers, which maintain the genetic integrity of co-existing *Daphnia* taxa. Thus far, no quantitative data are available on the extent of sexual reproduction among *Daphnia* hybrids and their parental species. Furthermore, it is not clear which role back-crossed individuals play in sexual reproduction and how back-crosses are distributed over hybrid and parental taxa. In the *Daphnia galeata–hyalina–cucullata* hybrid species complex two species-specific allozyme markers are known. Until recently researchers used the allozyme locus *AAT* [aspartate aminotransferase also referred to as *GOT* in earlier publications (Wolf and Mort, 1986; Wolf, 1987; Weider and Stich, 1992)] to identify parental species and hybrids, but Gießler (Gießler, 1997a) using laboratory clones, found that the allozyme locus *AO* (aldehyde oxidase) might be an even better species-specific marker. Since individual daphnids can be analysed for both enzymes it is possible to quantify potential back-cross individuals in field surveys. However, two species-specific markers allow only for the detection of 50% of the back-crosses, since the other offspring will have either the parental or the F_1 hybrid genotype. Apart from allozymes, several molecular species-specific markers are available for the *Daphnia galeata–hyalina–cucullata* hybrid species complex. Since DNA isolation and polymerase chain reaction studies involving many thousands of animals can be cost-prohibitive, allozyme electrophoresis is still the method of choice for temporal population studies that require large sample sizes.

Some studies have highlighted the importance of post-mating barriers by hybrid breakdown, i.e. decreased viability or fertility of back-cross hybrids (Schwenk *et al.*, 2001). However, pre-mating barriers might also play an important role in decreasing gene flow among *Daphnia* taxa. For example, interspecific variation for the induction of male and sexual female production within *Daphnia* species and their hybrids has been observed (Spaak, 1995), which might cause the temporal separation of sexually-reproducing *Daphnia* taxa. Also behavioural differences between taxa can result in pre-mating barriers. Differential diel vertical migration behaviour of parthenogenetic females of various *Daphnia* taxa is a well-known mechanism to avoid visually hunting predators (Lampert, 1993). Especially large *Daphnia* taxa, e.g. *D. hyalina* in Lake Constance (Weider and Stich, 1992), tend to migrate to the deeper and darker waters of a lake during the day and come to the surface to feed during the night, whereas other daphnids stay in the food-rich upper water layers, which poses a high predation risk all the time, e.g. *D. galeata* in Lake Constance (Weider and Stich, 1992). Moreover, experimental evidence exists for differential distributions of both sexes within a species, i.e. *Daphnia pulicaria* (Brewer, 1998). Brewer (Brewer, 1998) observed that male *D. pulicaria* stayed above the thermocline, whereas females remained in regions with the highest food concentration. Different migration strategies between, as well as within, species might function as a pre-mating reproductive isolating barrier between the taxa of the *Daphnia galeata–hyalina–cucullata* hybrid species complex.

The main goal of our study was to obtain better insight into the genetic differentiation of sexual and asexual forms of co-occurring *Daphnia* parental species and hybrids. We were especially interested in the occurrence and temporal distribution of possible back-crosses. Furthermore, we wanted to find out if sexual forms of different taxa co-occur in time and/or space to elucidate if recent

hybridization events are plausible. Therefore, we examined differences in spatial and temporal patterns of sexually-reproducing genotypes of *Daphnia* throughout one growing season. We investigated whether there are different distinct sexual phases within the *Daphnia galeata–hyalina–cucullata* hybrid species complex in spring and autumn, which could serve as a temporal reproductive barrier. Based on previous work (Weider and Stich, 1992) that has shown differential vertical migration strategies among parthenogenetic individuals of different taxa in this complex, we examined whether sexual morphs (i.e. ephippial females, males) show comparable vertical distributions that could indicate potential sexual reproduction between taxa.

METHOD

Study area

The *Daphnia* community was studied in the Plußsee, a small funnel-shaped lake (14 ha, maximum depth 29 m) (Overbeck and Chróst, 1994) in northern Germany. During the summer, this eutrophic lake shows a stable stratification, which is characterized by a thin (only a few metres deep) oxygen-rich epilimnion and a large anoxic, H_2S-rich hypolimnion beginning at a depth of 5–8 m. During spring and autumn the lake is completely mixed. The phytoplankton community in the lake shows a seasonal succession of diatoms, cyanophytes and chlorophytes (Overbeck and Chróst, 1994). There is also a seasonal succession in the zooplankton community, with copepods dominating during spring, daphnids in early summer, followed by the smaller cladocerans, *Diaphanosoma* and *Ceriodaphnia*, in autumn. The main predators of cladocerans in the Plußsee are phantom-midge larvae (*Chaoborus*), perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*). The coexistence of three *Daphnia* species (*D. galeata*, *D. cucullata*, *D. hyalina*) and their interspecific hybrids (*D. galeata* × *hyalina*, *D. cucullata* × *galeata*, *D. cucullata* × *hyalina*) was first described by Wolf and Mort (Wolf and Mort, 1986).

Field methods

To follow seasonal changes within the *Daphnia* community, plankton samples were taken with two vertical (Wisconsin-style) plankton net hauls through the entire water column (20 m) every week starting in April 1997 until December 1997. An additional sample was taken in February 1998. The samples were taken at two different sites in the middle of the lake. One-hundred and twenty female daphnids carrying eggs and 60 males and 60 ephippial females (if present) were randomly collected per sampling day and used for the genetic analysis. The rest of the sample was preserved in 95% ethanol and the total number of daphnids was counted to estimate population densities. Every 2 weeks from May to October 1997, additional samples were collected at different depths (1, 3, 5, 7, 12 and 20 m) during the day (11:30 h) and night (23:30 h) using a 30.5 L Schindler trap (Schindler, 1969). To decrease sampling variability because of zooplankton patchiness, depth series at two different sites were collected on each sampling date, and then pooled. At each depth, where possible, 60 egg-carrying females, 60 males and 60 ephippial females were randomly collected, and used for genetic analysis. The rest of the sample was stored in 95% ethanol and counted later.

Genetic structure

Cellulose acetate electrophoresis (Hebert and Beaton, 1989) was used to determine taxon affinity, as well as to identify distinct genotypes within taxa, using four different enzymes [*AAT*, EC 2.6.1.1; phosphoglucoisomerase (*PGI*), EC 5.3.1.9; phosphoglucomutase (*PGM*), EC 5.4.2.2; *AO*, EC 1.2.3.1]. Previous studies have shown that *AAT* is a reliable taxon-specific marker by which to identify daphnids from the Plußsee (Wolf and Mort, 1986). Therefore *AAT* was used as a fixed marker to determine taxon identity. *Daphnia galeata* is homozygous for the fast (F) allele, *D. hyalina* is homozygous for the slow (S) allele, and *D. cucullata* is homozygous for the very slow (S^-) allele. Heterozygous individuals indicate hybrids (e.g. *D. galeata* × *hyalina* has the *AAT* SF genotype). Recent studies suggest that *AO* is a more reliable marker than *AAT* within the *Daphnia galeata–hyalina–cucullata* hybrid species complex in southern Germany (Gießler, 1997a). Consequently *AO* was included in our study to quantify possible back-crosses, as well as *PGI* and *PGM*, which have been used previously as polymorphic enzymes to discriminate between various clones within this complex (Wolf, 1987; Weider and Stich, 1992; Spaak, 1994; Müller and Seitz, 1995). For brevity, we use the term MultiLocus Genotype (MLG) to designate distinct MLGs with the understanding that a given MLG may actually represent a clonal group, which may range from one to many clonal lineages (Weider, 1984). The number of individuals of a certain taxon (MLG) at a certain day and/or depth was calculated by adding the electrophoretically analysed animals to the ethanol counts (the other fraction of the sample). These totals (juveniles and adults) were multiplied by the clonal fractions as determined with electrophoresis to calculate the number per litre per taxon (sex) per date per depth.

To calculate genetic diversity, Simpson's index of concentration, (Simpson, 1949) $\lambda = \Sigma p_i^2$, was used, where p_i represents the frequency of the ith MLG in the sample. Clonal diversity was calculated as $D = -\log \lambda$ (Pielou, 1975). Thus D indicates the relative abundance of MLGs: low values of D indicate that a single clone is dominant,

while high values indicate that many clones are abundant at approximately equal frequencies (Spaak, 1994). A row × column test of independence (*G*-test; Sokal and Rohlf, 1995) was used to test if males, sexual females and parthenogenetic females were distributed differently in the water column during day and night.

RESULTS

Seasonal abundance of sexually-reproducing *Daphnia*

Based on the *AAT* electromorphs, all six taxa of the *Daphnia galeata–hyalina–cucullata* hybrid species complex (*D. galeata* – FF; *D. hyalina* – SS; *D. cucullata* – $S^{-}S^{-}$; *D. galeata* × *hyalina* – SF; *D. cucullata* × *galeata* – $S^{-}F$; *D. cucullata* × *hyalina* – $S^{-}S$) were detected in the Plußsee during the 1997/98 field season (Figure 1). Prior to a midsummer die-off of the entire *Daphnia* population, the hybrid *D. galeata* × *hyalina* was the most abundant taxon. During September, population densities recovered, although at a much lower level, with *D. hyalina* being the dominant taxon. *Daphnia galeata* and *D. cucullata* × *galeata* were mainly abundant in early summer, whereas *D. cucullata* and *D. cucullata* × *hyalina* were found, albeit only in very low numbers, throughout the entire year. Sexually-reproducing animals were found, with the exception of June 12, only during autumn and early winter, with a 2 week time lag between the first occurrence of males and the occurrence of ephippial females. At that time, up to 50% of the entire *Daphnia* population consisted of sexual morphs. Most of these animals were identified as *D. hyalina* (Figure 2), followed by the hybrid *D. galeata* × *hyalina* (Figure 3), with both *D. galeata* and *D. cucullata* found only at very low densities (Figure 1, Table I).

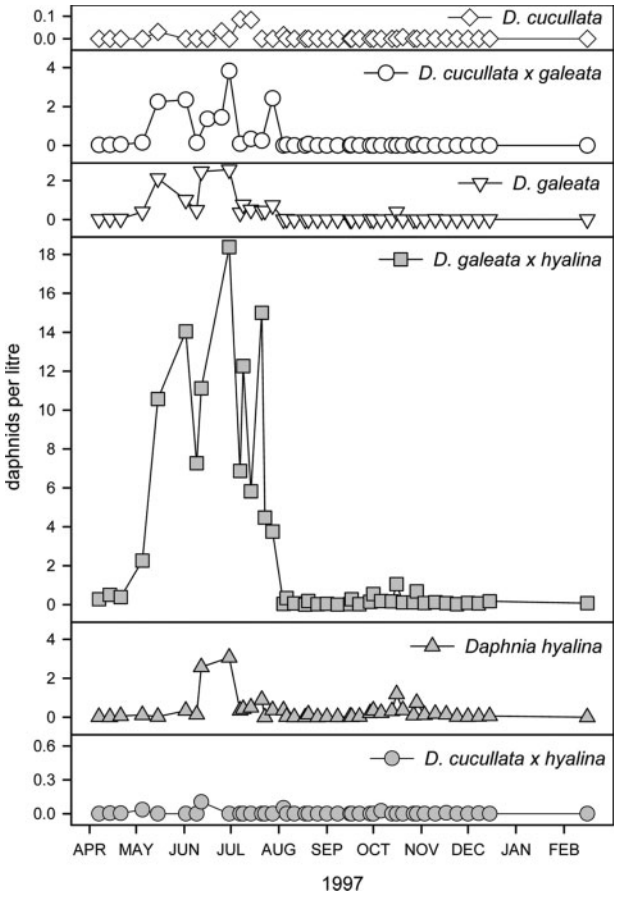

Fig. 1. Seasonal abundance of different adult parthenogenetic *Daphnia* taxa in the Plußsee during the study period (April 1997 to February 1998). Density estimates (based on ethanol-preserved specimens) were calculated for each taxon according to *AAT* genotype proportions (see text). *Daphnia cucullata* × *hyalina* and *D. cucullata* were detected only at very low densities throughout the year, and are therefore plotted on a different scale. Ticks mark the first day of a month.

Genotype frequencies and clonal diversity

The genotypic composition of the *Daphnia* population in the Plußsee in 1997/98 was very much influenced by a dramatic decline in population densities in August 1997 (very few daphnids were found in the lake during this time). The formerly dominant hybrid *D. galeata* × *hyalina* showed only a minor shift (compared to *D. hyalina*) in its genetic composition (Figure 3), whereas *D. hyalina* (Figure 2) switched its clonal diversity pattern completely from a population made up of numerous MLGs, to one dominated by a single MLG (no. 026). This MLG was not detected in the lake prior to the population crash in August but after the summer rebound it became the dominant MLG not only within the parthenogenetic fraction of the *D. hyalina* population, but also within the sexual portion (i.e. males and ephippial females). The dominance by this single MLG is also reflected by the very low clonal diversity estimates within sexual and asexual *D. hyalina* (Table II). In contrast to *D. hyalina*, where the most abundant parthenogenetic MLGs also exhibited all sexual morphs (Figure 2), three MLGs of the hybrid *D. galeata* × *hyalina* produced most of the males and ephippial females (Figure 3). These sexually-reproducing MLGs of *D. galeata* × *hyalina* represented only ~20% of the parthenogenetic population. Therefore, although the parthenogenetic hybrids continuously displayed high clonal diversity, *D*-values for sexually-reproducing animals were greatly decreased (Table II). Within the hybrid taxon *D. galeata* × *hyalina*, 85 distinct MLGs were found, while for *D. cucullata* × *galeata* 60 distinct MLGs were found throughout the sampling period. Numbers of *D. cucullata* × *galeata* were too low to calculate monthly *D*-values.

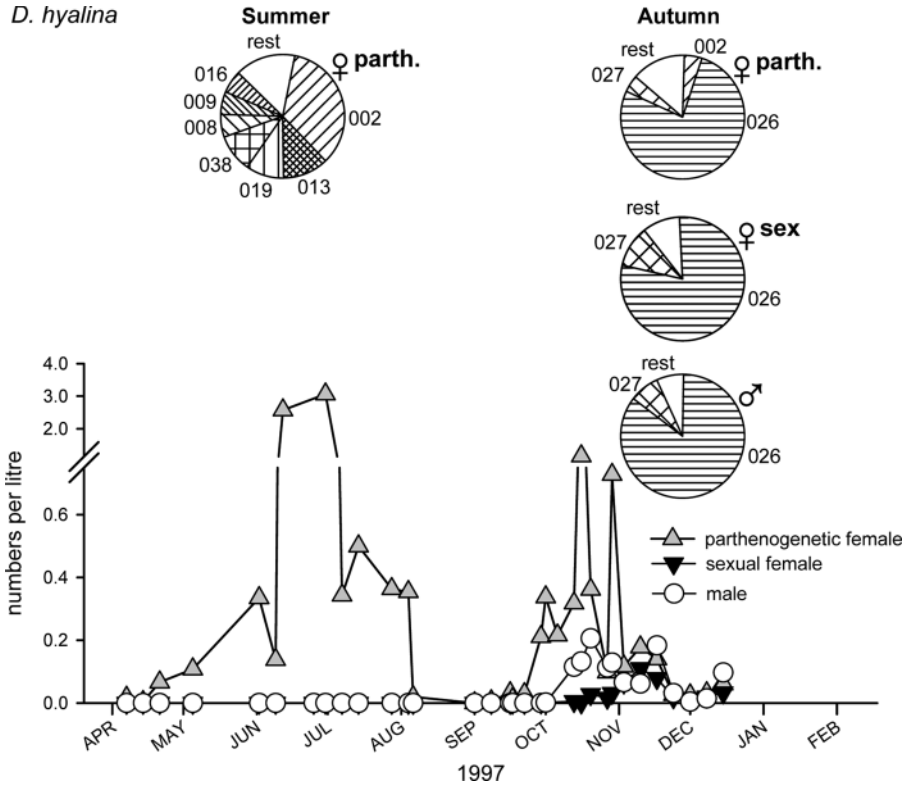

Fig. 2. Densities and clonal composition of parthenogenetic females as well as males and ephippial females of *Daphnia hyalina* in the Plußsee. In the pie diagrams, only MLGs (indicated with three-digit numbers) with an abundance >4% are plotted for the 'Summer' (April 7 to August 1) and Autumn (September 29 to December 15).

Back-crossing

Three *AO* alleles, S, M, and F, were found within the *Daphnia* population of the Plußsee. From earlier work (Gießler, 1997a) it is known that the M and F alleles are specific for *D. galeata*, and various S alleles are specific for *D. cucullata* and *D. hyalina*. Unfortunately it was not possible to distinguish between these various S alleles in our field samples. This means that the S allele scored here represents *D. cucullata* as well as *D. hyalina* alleles. Based on *AAT*, however, *D. cucullata* densities seem to be very low in the Plußsee, therefore, the analysis is concentrated on the most common taxa *D. hyalina*, *D. galeata* and their hybrid. If individuals show for one species-specific marker a homozygote and for the other a heterozygote pattern (for example *D. hyalina AAT*: SS, *AO*: SF) then is this an indication of a cross within a hybrid or of a back-cross of a hybrid with one of the parental species. For the parthenogenetic *D. hyalina* (all taxa names based on *AAT* genotype) in the Plußsee all possible *AO* genotypes were found, 29% of the animals had an *AO* genotype different from SS and should be considered as back-crosses (Figure 4, Table III). The males and ephippial females of *D. hyalina*, however, constituted >98% of 'pure' animals all with an *AO* genotype of SS. Of the parthenogenetic *D. galeata* × *hyalina* 16% of the *AO* genotypes were not 'hybrid-like', 9% had a *D. hyalina* genotype (SS) and 7% a *D. galeata* genotype (MM, MF, FF) (Figure 4). The sexual *D. galeata* × *hyalina* individuals almost all had a back-cross genotype, 97% of the males and 91% of the ephippial females were SS at *AO*. Not many *D. galeata* were found, the pattern within the parthenogenetic females was comparable to the *D. galeata* × *hyalina* hybrids, 61% had a 'pure' genotype. Only one *D. galeata* male and seven ephippial females were found.

Spatial patterns

Because of very low densities or the complete absence of males and/or ephippial females during most of the year (Figures 2 and 3), the vertical distributions of co-occurring parthenogenetic and sexual *Daphnia* in the water column could only be examined for October 1997 (Figure 5). Our data indicate that the vertical distributions of parthenogenetic *D. galeata* × *hyalina* and *D. hyalina* differed significantly between day and night (Table IV), but for the sexual animals the only significant differences found were for *D. hyalina* males between day and night, which indicates

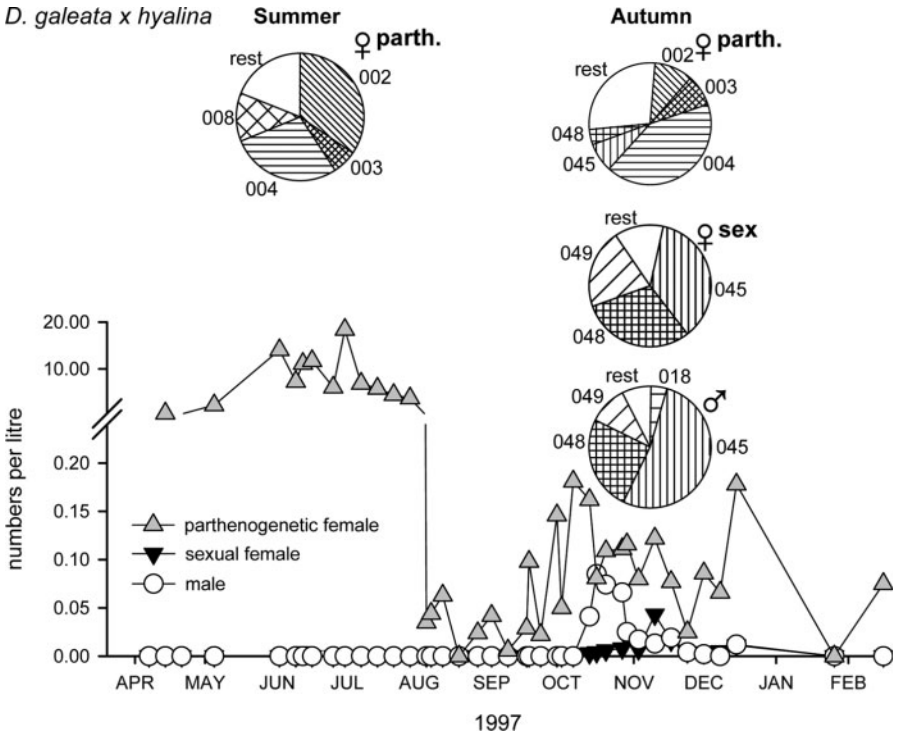

Fig. 3. Densities and clonal composition of parthenogenetic females as well as males and ephippial females of the *Daphnia galeata* × *hyalina* in the Plußsee. In the pie diagrams, only MLGs (indicated with three-digit numbers) with an abundance >4% are plotted for the 'Summer' (April 7 to August 1) and Autumn (September 29 to December 15).

Table I: Number of electrophoretically (AAT, see text) identified male (M) and ephippial females (E) per date

Date	*D. cuc.*		*D. cuc.* × *gal.*		*D. cuc.* × *hyl.*		*D. gal.*		*D. gal.* × *hyl.*		*D. hyl.*	
	E	M	E	M	E	M	E	M	E	M	E	M
12 Jun 97							10		4		1	
29 Sep 97											1	
1 Oct 97												3
13 Oct 97							1	2	3	25	4	54
16 Oct 97		1					1	2	14	41	4	59
20 Oct 97	1	3							6	18	20	38
27 Oct 97		1		1		1		4	8	35	12	45
29 Oct 97		2				3	2	3	50	85	64	227
3 Nov 97	1								7	12	48	34
10 Nov 97		1		1			2		16	7	31	24
17 Nov 97	1				3			1	16	14	59	102
24 Nov 97									4	1	13	7
1 Dec 97							1		3	2	11	3
8 Dec 97									5		15	11
15 Dec 97								1	11	12	25	72
Total	3	8	0	2	3	4	17	13	147	252	308	679

Only sampling dates are listed in which at least one male or ephippial female could be analysed. *D. cuc.*, *Daphnia cucullata; D. cuc.* × *gal.*, *Daphnia cucullata* × *galeata; D. cuc.* × *hyl.*, *Daphnia cucullata* × *hyalina; D. gal.*, *Daphnia galeata; D. gal.* × *hyl.*, *Daphnia galeata* × *hyalina; D. hyl.*, *Daphnia hyalina*.

Table II: Clonal diversity D calculated per month as the negative logarithm of Simpson's index of concentration (see text) for parthenogenetic females, males and ephippial females of Daphnia hyalina and D. galeata × hyalina

Month	*D. hyalina*			*D. galeata × hyalina*		
	part	male	ephippial	part	male	ephippial
Apr 97	0.816 (31)			0.482 (414)		
May 97				0.550 (608)		
Jun 97	0.977 (67)			0.656 (1022)		
Jul 97	0.398 (64)			0.254 (1026)		
Aug 97	0.760 (48)			0.464 (69)		
Sep 97	0.202 (101)			0.490 (162)		
Oct 97	0.231 (413)	0.144 (426)	0.276 (104)	0.769 (243)	0.423 (204)	0.437 (81)
Nov 97	0.495 (45)	0.150 (167)	0.163 (151)	0.719 (86)	0.376 (34)	0.621 (43)
Dec 97		0.101 (86)	0.227 (51)	0.476 (153)		
Jan 98						
Feb 98				0.416 (54)		

Sampling dates with less than 30 individuals per group (parthenogenetic females, males and ephippial females) were excluded. Sample sizes between brackets.

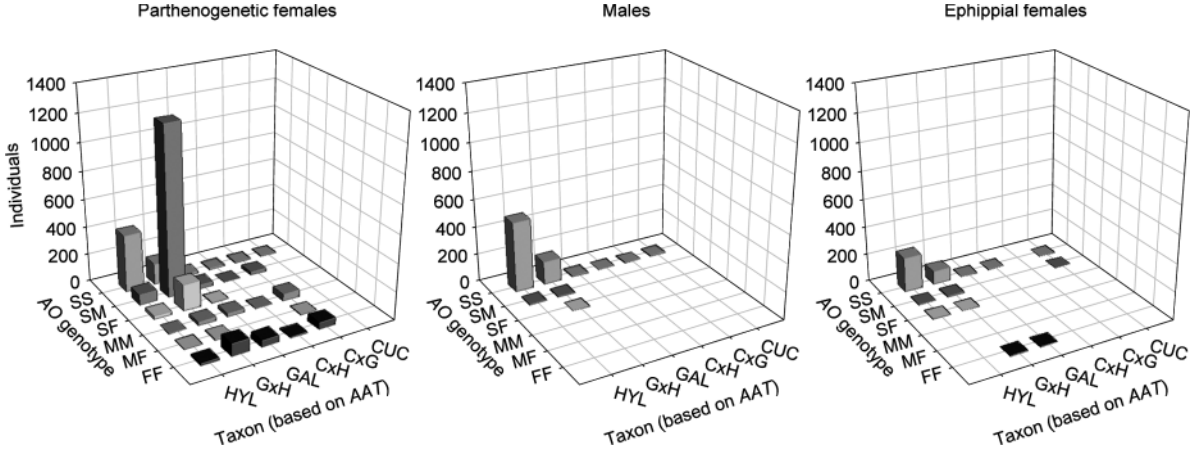

Fig. 4. Distribution of *AO* genotypes for the six *Daphnia* taxa found in the Plußsee. The numbers indicate the number of analysed individuals for the whole study period. On the *AAT* axis *D. hyalina* (HYL), *D. galeata* × *hyalina* (G×H), *D. galeata* (GAL), *D. cucullata* × *hyalina* (C×H), *D. cucullata* × *galeata* (C×G) and *D. cucullata* (CUC) are listed based on their electrophoretic genotype. For the *AO* genotype it should be noticed that FF (homozygote for the F-allele) indicates *D. galeata*, whereas MM and SS indicate *D. cucullata* and *D. hyalina*, respectively. The hybrids are defined as the heterozygotes.

Table III: Genotypes of the most frequent Daphnia hyalina and D. galeata × hyalina multi-locus genotypes (MLGs) in the Plußsee (see Figures 2 and 3)

MLG	*AAT*	*AO*	*PGI*	*PGM*
Daphnia hyalina				
002	**SS**	**SM**	**MF**	**FF**
008	**SS**	**SF**	**MM**	**MF**
009	**SS**	**SM**	**MM**	**FF**
013	**SS**	**SM**	**MM**	**MF**
016	**SS**	**SF**	**MF**	**FF**
019	**SS**	**SM**	**MM**	**MF$^+$**
026	SS	SS	MM	FF
027	SS	SS	MM	FF$^+$
038	**SS**	**FF**	**MM**	**MF$^+$**
057	**SS**	**MM**	**SF**	**MM**
Daphnia galeata × hyalina				
002	SF	SM	MM	MF
003	SF	SF	MM	MF
004	SF	SM	MF	FF
008	SF	SM	MM	MF$^+$
018	**SF**	**SS**	**MM**	**MF**
045	**SF**	**SS**	**MM**	**FF**
048	**SF**	**SS**	**MM**	**FF$^+$**
049	**SF**	**SS**	**MM**	**FF^{++}**

Genotypes in bold type indicate back-crosses.

that those animals migrate. There was a tendency for ephippial females to be higher in the water column at night compared to parthenogenetic females and males (Figure 5). This tendency, however, was not significant.

DISCUSSION

In this study, it is shown that the clonal composition of co-occurring sexual and parthenogenetic *D. galeata* × *hyalina* can differ. Furthermore, we show that after a mid-summer die-off the genetic composition within *D. hyalina* completely changed. Moreover, our data show the co-occurrence of three parental taxa and their hybrids within the same body of water from spring 1997 to spring 1998. Although clonal diversity varied within the taxa, the high number of distinct MLGs (85 for *D. galeata* × *hyalina* and 60 for *D. cucullata* × *galeata*) strongly implies multiple hybridization events, as has been suggested previously (Spaak, 1997). Our data also show that back-crossing events are frequent, although not all back-cross individuals seem to have an equal chance to reproduce sexually.

Genetic polymorphism and back-crossing

After the summer die-off of the *Daphnia* population, the same *D. galeata* × *hyalina* MLGs re-appeared in the lake as were present in the spring. For *D. hyalina*, however, the rebounding population was dominated by an MLG

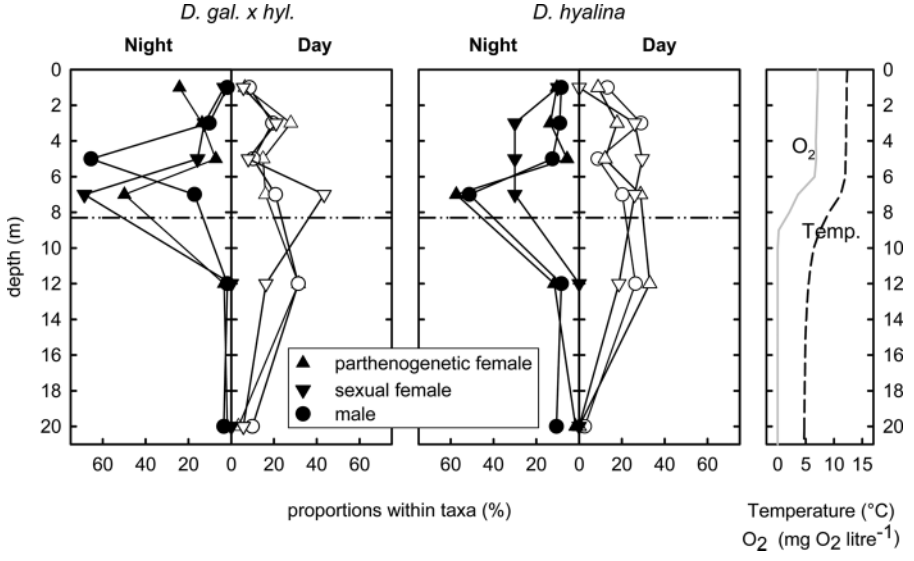

Fig. 5. Proportional depth distributions of parthenogenetic females, males and sexual (ephippial) females of *D. galeata* × *hyalina* and *D. hyalina* during the day (11:30 h) and night (23:30 h) in October 1997. Average temperature and oxygen gradients in the Plußsee during October are shown. Filled symbols indicate night distributions; open symbols indicate day distributions.

Table IV: Results of the row × column test of independence (G-test) of differences in distribution between day and night of pooled October 1997 samples of daphnids sampled at six different depths (Figure 5)

Taxon	*G*-test	d.f.	*P*
Daphnia hyalina			
Parthenogenetic	41.49	5	<0.001
Sexual female	2.28	5	NS
Male	11.69	5	<0.05
Daphnia galeata × hyalina			
Parthenogenetic	53.20	5	<0.001
Sexual female	3.15	5	NS
Male	6.29	5	NS

that had not been found prior to the die-off (Figure 2), suggesting that this MLG either hatched from ephippia, or was found at such low densities prior to August 1997 that it escaped detection. Furthermore, the agreement with Hardy–Weinberg expectations of the genotype frequencies found in autumn (data not shown) supports the hypothesis of a hatching event in the Plußsee in autumn 1997. It is remarkable that the *D. hyalina* MLGs before the summer die-off all consisted of back-cross genotypes whereas the autumn MLGs (including the males and ephippial females) were all 'pure' *D. hyalina* with an *AO* SS genotype (Figure 2; Table III). Also the hybrid *D. galeata* × *hyalina* males and ephippial females all had an *AO* SS genotype. This suggests that not all *Daphnia* genotypes reproduce sexually with the same probability. The fact that for *D. hyalina* the 'pure' and for the hybrid the back-cross to *D. hyalina* reproduces sexually might be an explanation why this species is still distinct despite their ability to hybridize and back-cross.

Very little is known about the timing of ephippial hatching within the *D. galeata–hyalina–cucullata* hybrid species complex. Only a few field studies (Wolf and Carvalho, 1989; De Stasio, 1990; Cáceres, 1998; Jankowski, 2002) have examined this extensively. Wolf and Carvalho (Wolf and Carvalho, 1989) used hatching traps to show that only a short and distinct period of ephippial hatching occurs in north German lakes in spring; they found no evidence for continuous hatching throughout the year. Interestingly, in our study, we found that the *D. hyalina* MLG that appeared after the summer decline, and most probably hatched from a diapausing egg, also produced the highest numbers of males and ephippial females. This suggests that this specific MLG might be adapted to reverting to sexual reproduction. In contrast, only a low percentage of the *D. galeata* × *hyalina* MLGs reproduced sexually (Figure 3), which might suggest decreased sexual fertility of the hybrid.

Spatial separation

Daphnia taxa (Stich and Lampert, 1981; Weider and Stich, 1992; Lampert, 1993) as well as clones within taxa (De Meester *et al.*, 1995) are known to show differences in their diel vertical migration patterns. These different depth preference and migration behaviours, as shown for example for *D. galeata* and *D. hyalina* in Lake Constance (Stich and Lampert, 1981; Weider and Stich, 1992) could also lead to reproductive isolation between *Daphnia* taxa, since sexual individuals would have a low encounter probability. In the Plußsee, the mean depths in the water column of parthenogenetic *D. hyalina* and *D. galeata* × *hyalina* were the same. However, a row × column (*G*-test) of independence showed significant differences ($G_{day} = 20.15$, $P < 0.01$; $G_{night} = 14.39$; $P < 0.05$) indicating that the distributions of both taxa differ. Comparing sexual individuals between taxa showed no significant differences.

To our knowledge, our paper presents the first detailed field data about the vertical distributions of *Daphnia* males and ephippial females in this hybrid species complex. Ephippial females of *D. galeata* × *hyalina* were concentrated just above the thermocline during the night, where *D. hyalina* males were also found; *D. galeata* × *hyalina* males and *D. hyalina* ephippial females stayed closer to the surface (i.e. around 5 m, Figure 5) during the night. During the day, males and ephippial females were spread more throughout the water column (Figure 5). Ephippial females of *D. galeata* × *hyalina* and *D. hyalina*, however, exhibited their highest densities just above the thermocline and were somewhat higher in the water column compared to the parthenogenetic females, as was found by Spaak and Boersma (Spaak and Boersma, 2001). But these differences were not significant using a row × column (*G*-test) of independence (Table IV). These results are counter-intuitive as sexual females are more conspicuous than parthenogenetic females; one might expect a stronger diel vertical migration behaviour for sexual forms. A possible explanation might be that the sexual females prefer the warmer water where their sexual eggs will develop more quickly.

Behavioural experiments in 1 m tall vertical migration chambers have revealed different distribution patterns of male and female *Daphnia* (Brewer, 1998). Males stayed just above the thermocline, whereas ephippial females stayed in the upper part of the water column where both temperature and food levels were higher. The vertical distribution of males in the Plußsee differs from the experimental results on *D. pulicaria* where males and

ephippial females were more separated (Brewer, 1998). Our results show that males are deeper in the water column during the night, but during the day distributions of both sexes fully overlap. This indicates that, despite earlier laboratory experiments (Brewer, 1998), we found no evidence for behavioural pre-mating barriers within this species complex.

Temporal separation

Two taxa (*D. hyalina* and *D. galeata* × *hyalina*) produced significant numbers of males and sexual females in October and November 1997; no sexual forms were observed in August, when the population collapsed. In October and November, males and ephippial females of *D. galeata* and *D. cucullata* were also found, although in very low numbers. Earlier studies have shown a temporal separation between the periods of sexual reproduction of *D. galeata* and *D. hyalina*, (Wolf, 1987) as well as *D. galeata* and *D. cucullata* (Spaak, 1995; Schwenk, 1997) in the field. According to these earlier findings, *D. galeata* reproduces sexually mostly in the spring in north temperate European lakes, whereas *D. hyalina* and *D. cucullata* exhibit sexual morphs in autumn. Additional laboratory experiments have detected large inter- and intraclonal variation in receptivity to environmental stimuli (i.e. photoperiod, shifts in population density, presence of food or predators) (Spaak, 1995) that induce sexual reproduction. Contrary to the findings of Wolf (Wolf, 1987) and Spaak, (Spaak, 1995) our study did not detect a separate extensive sexual phase of *D. galeata* in early summer, when only very few ephippial females of *D. galeata* were caught in the Plußsee. Extraordinary climatic conditions (i.e. very compressed epilimnion, heavy H_2S-rich hypolimnion because of the long winter 1996/97), resulting in the absence of a refugium for fish predation, might be the cause of the very rapid August 1997 decline in the *Daphnia* population (i.e. complete population crash within 2 days) and therefore the absence of sexual *Daphnia* in this period. The simultaneous presence of sexual morphs of various taxa in autumn indicates that back-crossing and hybridization have the potential to occur in the Plußsee.

In conclusion, our study has shown that sexual forms of representative taxa in the *Daphnia galeata–hyalina–cucullata* hybrid species complex co-occur in the Plußsee. This co-occurrence provides the opportunity for the continuous production of hybrids and back-crosses. On the other hand, we could show that only specific genotypes produce males and sexual females. Moreover, no sexual individuals of *D. galeata* and *D. cucullata* × *galeata* were found although parthenogenetic females were present in reasonable numbers during spring. This suggests that pre-mating barriers exist, to a certain extent, between the *Daphnia* taxa in this lake, as was expected based on laboratory experiments (Spaak, 1995). We could however, find no evidence for spatial segregation of sexual forms of different taxa, as was hypothesized based on DVM studies of parthenogenetic females. Stronger evidence was found for post-mating barriers, only hybrid genotypes that are back-crosses to the parental (*D. hyalina*) species seem to reproduce sexually, indicating a lower fitness for regular hybrids, moreover, only 'pure' *D. hyalina* genotypes produced sexual forms and not the back-cross forms. The combination of genetic factors, as well as the different timing of sexual reproduction, seem to be the reasons why the individual species in this complex still exist.

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