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Intrinsic postzygotic barriers constrain cross-fertilisation between two hybridising sibling rotifer species of the *Brachionus calyciflorus* species complex

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Abstract

1. *Brachionus calyciflorus* is a recently described monogonont rotifer species complex that comprises four species. The observation of hybridisation between two of these species challenges this species delimitation. The mechanisms of reproductive isolation essential to the maintenance of species integrity remain unclear.
2. Here, we conducted upscaled hybridisation experiments to obtain large numbers of hybrid and non-hybrid dormant propagules. Through hatching assays, we compared the zygote viability of hybrid with non-hybrid dormant propagules. Furthermore, we investigated populations of F1 hybrid clones and assessed their clonal growth rate and the ability to reproduce sexually.
3. Our results demonstrated higher rates of morphological abnormality and associated mortality in dormant propagules of hybrids compared to non-hybrids yet hatching rates of healthy-looking propagules proved similar. F1 hybrids exhibited high clonal population growth rates, nevertheless, we also observed strong differences between clones and a strong influence of parental genotype identity. Two-thirds of the F1 hybrid clones showed a low incidence of sexual reproduction and almost never produced dormant propagules. Clones with high population growth rates seemed to invest less in sexual reproduction.
4. Our results clearly demonstrate the existence of intrinsic postzygotic barriers caused by relatively high mortality of dormant propagules. Furthermore, the low ability of most hybrid clones to engage in sexual reproduction may reduce the long-term fitness of hybrid clones. These postzygotic barriers probably impede genetic exchange between parental species and contribute to the maintenance of their integrity.

KEYWORDS
cross-mating, integrative taxonomy, introgression, monogonont rotifers, reproductive isolation

1 | INTRODUCTION

The process of speciation is usually driven by the emergence and subsequent reinforcement of reproductive barriers. Upon secondary contact of incipient species (Cenzer, 2016; Comeault et al., 2015), the strength of reproductive barriers will determine whether species boundaries are maintained (Martin & Willis, 2007; Tellier et al., 2011) or diverging populations fuse again into a single lineage (Kearns et al., 2018; Todesco et al., 2016). Alternatively, there are cases where hybridisation and admixture are an ongoing process while species boundaries continue to persist (Haselhorst et al., 2019; Ma et al., 2019; Peek et al., 2019). Therefore, the study of the magnitude of reproductive barriers is crucial to understand the stage of speciation and predict the fate of hybridising species.

Reproductive isolation between sibling species is often the consequence of multiple isolating barriers (Coyne & Orr, 2004) that can act before or after the formation of a zygote. However, the relative strengths of such pre- and postzygotic barriers are variable among different species pairs (Johnson et al., 2015; Sánchez-Guillén et al., 2014) and their relative role is probably dynamic also throughout the speciation process (Kulmuni et al., 2020). Prezygotic barriers are often considered the most important barriers because they act ahead of postzygotic barriers (Lackey & Boughman, 2017; Rull et al., 2013; Sánchez-Guillén et al., 2012). However, postzygotic barriers can play an essential role as well particularly when prezygotic barriers are permeable (Gebiola et al., 2016; Johnson et al., 2015; Rull et al., 2012). As a result of natural selection against inferior hybrids, postzygotic barriers can also result in the emergence and reinforcement of prezygotic barriers (Poikela et al., 2019; Rosser et al., 2019).

Intrinsic postzygotic barriers occur when the fitness of hybrids, regardless of the environment, is consistently reduced compared to that of either parental species, due to e.g. hybrid inviability or sterility. A number of mechanisms can give rise to intrinsic postzygotic barriers such as changes in ploidy (Wu et al., 2016), differences in endosymbionts (König et al., 2019), and meiosis defects due to substantial chromosomal divergence (Greig, 2009). More frequently, however, intrinsic postzygotic barriers evolve as a side-effect resulting from the accumulation of genetic substitutions in the populations of diverging parental taxa (i.e. the Dobzhansky–Muller model of genetic incompatibilities; reviewed in Coughlan & Matute, 2020). Upon secondary contact, inviable or sterile hybrids may be formed due to genetic incompatibilities (Brideau et al., 2006; Feng et al., 2020).

Intrinsic postzygotic barriers can contribute to speciation by reducing gene flow between diverging populations. Apparently, all kinds of reproductive barriers should be able to restrict introgression, but the efficacy of a specific barrier in preventing introgression may be influenced by many factors. For example, spatially isolated species can hybridise upon secondary contact (Schwarzer et al., 2012; Valencia-Montoya et al., 2020). Climate change is another important driver that may dissolve species boundaries maintained by prezygotic barriers such as habitat, time and behavior (reviewed in Chunco, 2014). Conversely, intrinsic postzygotic barriers that stem from genetic incompatibilities are independent of external environmental factors and are therefore more persistent in preventing the blending of species (Larcombe et al., 2016; Moran et al., 2019). In a recent simulation study of secondary contact between two incipient species, assortative mating was unable to maintain species boundaries whereas a small reduction in hybrid survival was able to substantially reduce hybridisation and introgression (Irwin, 2020).

Monogonont rotifers, for which at least 1,600 species have been described, are facultative sexuals. In the *amictic* or asexual life cycle, females produce exact genetic copies of themselves through ameiotic parthenogenesis. The sexual life cycle is initiated when chemical signals are produced that initiate the formation of *mictic* or sexual daughters (Snell et al., 2006). These sexual offspring will produce either haploid males (if not fertilised) or diploid dormant propagules (if fertilised at an early age). Dormant propagules can survive adverse periods and give rise to amictic females when conditions turn favourable. Unhatched dormant propagules accumulate in the sediment, forming dormant propagule banks (Serra et al., 2019).

Brachionus calyciflorus is arguably the most intensively studied freshwater monogonont rotifer. The taxon was first suggested to consist of a species complex by Gilbert and Walsh (2005). Evidence for interspecific divergence has since been suggested by a variety of studies based on molecular phylogenetic (García-Morales & Elías-Gutiérrez, 2013; Papakostas et al., 2016), ecological (Paraskevopoulou et al., 2018; Zhang et al., 2018; Zhang et al., 2019), and phylogeographic analyses (Xiang et al., 2011a, 2011b; Zhang, 2021), as well as experimental assessments of reproductive isolation between phylogenetically divergent clones (Gilbert & Walsh, 2005; Li et al., 2008; Xiang et al., 2011a, 2011b). Based on a comprehensive analysis of all publicly available sequences of the cytochrome c oxidase subunit I (COI) and the internal transcribed spacer I (ITS1), Papakostas et al. (2016) suggested the existence of four species. A morphometric study by Michaloudi et al. (2018) indeed demonstrated consistent morphometric differences which formed the basis of a formal (re-) description of four species, i.e. *B. calyciflorus* s.s., *Brachionus dorcas*, *Brachionus elevatus*, and *Brachionus fernandoi*.

Despite all this evidence, these species seem to consist of a hybridising species complex. Molecular analyses based on field surveys (Li et al., 2008; Papakostas et al., 2016; Zhang et al., 2018) report the occurrence of mitonuclear discordances between nuclear and mitochondrial markers among all four species. Additional analysis (Papakostas et al., 2016; Zhang, 2021) strongly suggests that these discordances originate from hybridisation and introgression rather than incomplete lineage sorting. Furthermore, in a short-term competition experiment, *B. calyciflorus* s.s. and *B. elevatus* were found to produce F1 hybrids and possible backcrosses within the time span of a month (Papakostas et al., 2016). Finally, Zhang et al. (2019) hatched *B. calyciflorus* s.s. × *B. elevatus* hybrid dormant propagules from lake sediments and proved able to establish viable cultures from them. Although the latter observations suggest that hybridisation is currently ongoing in natural populations, this idea is at odds with the results of a recent large scale phylogeographic analysis of mitonuclear discordance patterns within the species complex (Zhang, 2021),

which suggests that observed mitonuclear discordances date from postglacial range expansions or even before.

In another study (Zhang, 2021), we applied cross-fertilisation experiments and demonstrated that hybridisation between the sibling species *B. calyciflorus* s.s. and *B. elevatus* is hampered by strong yet penetrable prezygotic barriers. Although F1 hybrid dormant propagules were formed at low frequencies in both directions when males and mictic females from different species were combined, intraspecific fertilisation proved to occur much more frequently than interspecific fertilisation. Moreover, the chance that males were able to successfully fertilise females from the other species tended to be reduced in the presence of allospecific males. We also found evidence for a genetic component of fertilisation success given that some maternal clones proved to produce hybrid propagules in much higher frequencies than other clones. Despite such a prezygotic barrier, we were still able to produce some healthy-looking hybrid dormant propagules. However, due to the relatively small scale of these experiments, the numbers of produced hybrid dormant propagules were very small, preventing us from hatching and raising hybrid clones. This also precluded us from establishing their relative hatching success and any other intrinsic postzygotic barriers such as hybrid viability, growth performance, and sterility.

For this study, we set out to test for the existence of intrinsic postzygotic reproductive isolation barriers between *B. calyciflorus* s.s. and *B. elevatus*. We conducted an upscaled hybridisation experiment with the aims of obtaining a large number of both hybrid and non-hybrid dormant propagules that would allow us to estimate postzygotic mortality and to develop cultures of F1 hybrid clones for further study. In an effort to create hybrid dormant propagules, we exposed large numbers of juvenile mictic females to numerous males from the other species, involving multiple pairwise clone combinations. We then tried to hatch the resulting dormant propagules and applied microsatellite diagnostic markers to determine whether individual hatchlings were the product of interspecific or intraspecific fertilisation. In addition, we applied microsatellite analysis to a subset of unhatched dormant propagules to determine their hybrid status and to verify if there was a relationship between this status and the presence of morphological abnormalities indicative for propagule inviability. Finally, we established clonally reproducing populations from hybrid hatchlings and investigated their potential for clonal population growth and their ability to produce healthy-looking F2 dormant propagules.

2 | METHODS

2.1 | Sample collection, clone establishment, and stock culture maintenance

Sediments were collected from five sites in The Netherlands in April 2016 (Table 1). Dormant propagules of *B. calyciflorus* species complex were isolated by applying the sugar flotation method described by Gómez and Carvalho (2000). Dormant propagules were hatched under continuous light in petri dishes with demineralised water at room temperature. Dishes were checked at 12 hr intervals and hatched females were transferred individually to wells of 24-well plates filled with 1 ml of chemostat cultured *Chlamydomonas reinhardtii* (1,000 μmol C/L) resuspended in WC medium (Guillard, 1975). After population sizes had grown larger than five individuals, they were transferred to 20-ml plastic cups with 8 ml food and further maintained as stock cultures at room temperature under continuous light. Every other day, 10–15 females carrying parthenogenetic propagules were transferred to new cups with fresh food.

2.2 | DNA extraction, ITS1 polymerase chain reaction, restriction fragment length polymorphisms, and microsatellite genotyping

The species identity of stock cultures was identified using restriction fragment length polymorphisms together with microsatellite genotyping. First, DNA was extracted from a single rotifer using the HotSHOT method described by Montero-Pau et al. (2008). ITS1, between 296 bp and 313 bp, were amplified using the primers III: 5′-CACACCGCCCGTCGCTACTACCGATTG-3′ and VIII: 5′-GTGCGTTCGAAGTGTCGATGATCAA-3′ (Hwang et al., 2013). For restriction fragment length polymorphism analysis, 3 μl of ITS1 polymerase chain reaction products were digested by 1 μl *Alu*I and *Dra*I (New England Biolabs, Inc.) separately and incubated overnight at 37°C. *B. calyciflorus* s.s. and *B. elevatus* were identified by examining the pattern of DNA bands produced on 1% agarose gel as described by Papakostas et al. (2016).

Microsatellite analysis was applied to confirm the species identity of stock cultures (Table S1) and to assess the hybrid status of dormant propagules and cultures established from hatchlings. DNA for these analyses was extracted from single dormant propagules or rotifer individuals using the HotSHOT method (Montero-Pau et al.,

TABLE 1 Clones of *Brachionus calyciflorus* s.s. and *Brachionus elevatus* used in the upscaled hybridisation experiment with geographic coordinates of the sites where they were collected. Population ID: code of the population

Population ID	Latitude	Longitude	*B. calyciflorus* s.s.	*B. elevatus*
7	N 51.854065°	E 5.893175°	7_IIC	–
69	N 52.090694°	E 4.338444°	69_IC	69_IIIB
101	N 52.025980°	E 4.328860°	–	101_IIB
128	N 52.640324°	E 4.730287°	128_IIC	128_1B
168	N 51.491438°	E 4.306801°	168_IIC	–

2008). Twelve microsatellite loci were amplified using the primers described by Declerck et al. (2015). Alleles at loci A9 and A15 are known to be species specific (Papakostas et al., 2016) and were therefore used for species identification and the detection of F1 hybrids. Microsatellite analysis was conducted with an ABI Prism 3,130 DNA Analyzer (Applied Biosystems) and genotyping was done with GeneMapper v.5.0 software (Applied Biosystems).

2.3 | Experiments: general

All experiments were conducted in a food suspension of 1,000 μmol C/L food (*C. reinhardtii*) at room temperature (23 ± 1°C) and under continuous light.

3 | Preparation of animals for the upscaled hybridization experiment: induction of males and mictic females

For the upscaled hybridisation experiment, males and mictic females were induced by creating high-density cultures (Gilbert, 2007). Starting from animals from the stock cultures, populations were first grown in 500-ml flasks with abundant food (1,000 μmol C/L *C. reinhardtii*) at 24°C and under continuous light. Every other day, the food was refreshed by decanting approximately 80% of the culture and filtering it over a mesh (90 μm in case of high culture densities, 60 μm in case of low culture densities). Subsequently, animals were washed from the mesh and suspended in fresh food suspension. Part of each high-density culture was filtered through a 90-μm mesh to separate neonate females and males from adult females. Subsequently, neonate females were separated from males using a 60-μm mesh, whereas the remaining males were collected on a 30-μm mesh. Neonate females and males were rinsed into petri dishes, visually inspected, and immediately applied to the upscaled hybridisation experiments.

3.1 | Upscaled hybridisation experiment

We performed upscaled hybridisation assays by pooling large numbers of males and neonate females of the different species in 75-ml flasks with food suspension. Previous pilot experiments had shown that this approach yields both hybrid dormant propagules as well as dormant propagules produced through selfing of the mother clone, the latter being the result of fertilisation of mictic females in the preparatory phase and possibly during the hybridisation experiment as well. The simultaneous production of hybrid and non-hybrid propagules produced by the same maternal clone creates the opportunity to directly compare their viability and hatching success. From the 24 possible combinations (four clones of *B. calyciflorus* s.s. × three clones *B. elevatus* × two directions), 16 were tested. Given that five combinations were repeated more than once, this yielded a total of 24 assays (Table S2). In 11 assays, females of *B. elevatus* were pooled with males of *B. calyciflorus* s.s., whereas in the other 13 assays we combined *B. calyciflorus* s.s. females with *B. elevatus* males. After 5 days of incubation, dormant propagules were collected for further analysis.

3.2 | Collection, hatching, and microsatellite genotyping of dormant propagules produced in the experiments

A substantial fraction of dormant propagules was found to have an abnormal appearance (Figure S1). Abnormality in the shape of dormant propagules is known to be strongly associated with inviability. All dormant propagules produced in an assay were visually classified and sorted as *normal* (i.e. healthy-looking) or *abnormal* (i.e. with morphological aberrations) according to Gilbert (2010). Subsequently, they were separately incubated for hatching and monitored. Incubation took place at 24°C under continuous light in demineralised water. Propagules were checked every 12 hr for 5 days. Successfully hatched offspring were used to establish clonal lines. The hybrid status of non-hatched dormant propagules and successfully established clonal lines were determined with microsatellite analysis (Table S3 and S4). We did not include a dormancy period because propagules of *B. calyciflorus* have been shown to be able to hatch around 72 hr after their production (Stelzer, 2017). Furthermore, abnormal eggs were found to deteriorate quickly, hampering the identification of their hybrid status through microsatellite analysis.

Microsatellite analysis was performed on 418 non-hatched propagules (i.e. 206 normal and 212 abnormal dormant propagules) produced in six pairwise combinations of three clones in both fertilisation directions plus one additional combination (Table S4 and S5). For each combination, we aimed at analysing 30 normal (range 16–40) and 30 abnormal dormant propagules (range from 20 to 32).

3.3 | Characterisation of F1 hybrid clones

Of each of the 15 F1 hybrid clones obtained from the upscaled hybridisation experiment, we established a culture under standardised conditions for a period of more than 2 months. Populations were started with 10 individuals in 1 ml of food suspension. Every 2 days, we restarted populations by transferring 10 haphazardly selected females (without sexual propagules) to a fresh food suspension. After population growth had stabilised, we monitored the population for five consecutive 2-day time intervals by assessing the total number of females, the number of females with (normal and abnormal) dormant propagules and the number of females carrying unfertilised sexual eggs. For each time interval, we calculated the exponential population growth rate r of each of the hybrid clones as $(\ln N_t - \ln N_0)/t$, where N_0 and N_t represent the size of the populations at the start and end of the time intervals, respectively, and where t is the duration of the time interval.

TABLE 2 Time-averaged population growth rate, percentage of females with male and dormant propagules as well as the percentage of abnormal dormant propagules estimated from the 15 F1 hybrid clones obtained from the upscaled hybridisation experiment during a monitoring period of 10 days. *Brachionus elevatus*: 69_IIIB and 128_1B; *Brachionus calyciflorus* s.s.: 168_IIC and 69_IC

Mother clone	Father clone	Clone line no.	Population growth rate (day^{-1})	Percentage of females with unfertilised sexual eggs	Percentage of females with dormant propagules	Percentage of abnormal dormant propagules
168_IIC	69_IIIB	1	0.95	4.7%	–	–
		6	0.93	0.3%	–	–
		7	0.93	1.9%	–	–
		10	1.03	1.5%	0.3%	–
		12	0.97	2.0%	–	–
168_IIC	128_1B	8	0.74	3.3%	–	–
		9	0.73	4.0%	–	–
		11	1.04	0.7%	–	–
		12	0.98	1.7%	–	–
		14	0.98	3.1%	–	–
69_IC	128_1B	5	0.55	13.1%	12.4%	38%
		8	0.43	11.7%	14.2%	47%
		13	0.60	10.9%	12.1%	38%
		14	0.67	11.9%	10.3%	48%
		19	0.62	6.6%	15.3%	36%

3.4 | Statistics

Data were analysed with generalised linear models assuming a binomial distribution of the dependent variables and assuming a logit link function. Using data of the upscaled hybridisation experiment, we evaluated if the probability for dormant propagules to be abnormal differed between hybrid versus non-hybrid dormant propagules. For the data analysis of the F1 hybrid clone characterisation experiment, we compared populations of clones originating from different parental clone combinations regarding the frequency of females with dormant propagules and females with unfertilised sexual eggs in the populations by using sums of observations across time. In addition, *r*-values of each F1 hybrid clone were first averaged over time and used as replicate values to compare parental clone combinations using one-way ANOVA. In all other analyses, the significance of individual model terms was assessed by comparing alternative models as specified in Tables S6 using Akaike information criterion values and χ^2 difference tests. All statistical analyses were performed in the R software environment 3.6.3 (R Core Team 2020). Generalised linear models were performed using the lme4 package (Bates et al., 2014). Tukey post hoc tests were performed with the multcomp package (Hothorn et al., 2008).

4 | RESULTS

4.1 | Upscaled hybridisation experiment

The upscaled hybridisation experiment yielded a total of 4,067 dormant propagules of which 2,851 were normal and 1,216 were abnormal (Table S2). Within a hatching period of 5 days, 71 dormant propagules hatched and 35 offspring were successfully cultured (Table S2). Abnormal propagules never hatched. Microsatellite analysis revealed that 15 of these 35 clone lines were F1 hybrids (Table S3) that were uniquely produced by three parental clone combinations (168_IIC♀ × 69_IIIB♂, 168_IIC♀ × 128_1B♂, and 69_IC♀ × 128_1B♂). For each of these combinations, the cultures of five hybrid clonal lines were available for further investigation.

Microsatellite genotyping allowed us to successfully identify the hybrid status of 80% and 78% of the normal and abnormal dormant propagules, respectively (Table S5). Eighty of 165 (48%) of the successfully genotyped normal propagules were found to be hybrids, whereas the proportion of hybrids in abnormal propagules amounted to 71% (i.e. 118 on a total of 166). The share of abnormal propagules in the detected hybrids (60%) was therefore significantly higher than in non-hybrids (36%; $\chi^2(1) = 17.8$, $p < 0.001$; Table S6).

4.2 | Characterisation of F1 hybrid clones

Within the monitoring period, time-averaged exponential population growth rates *r* ranged from 0.43 to 1.04 day^{-1} across hybrid clones (Table 2). Population growth rates were strongly determined by the genotypes of the parental clones ($F(2, 12) = 19.9$, $p < 0.001$; Table S7) as the parental clonal combination 69_IC♀ × 128_1B♂ had substantially lower population growth rates than those produced by 168_IIC♀ × 69_IIIB♂ or 168_IIC♀ × 128_1B♂ (Tukey post hoc test, $p < 0.01$; Figure 1). Clone groups also

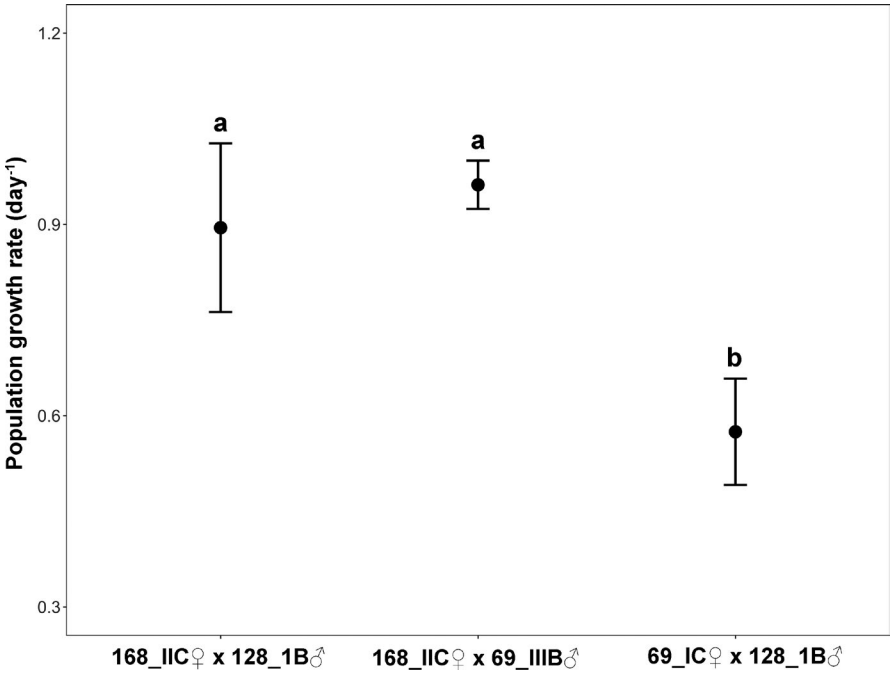

FIGURE 1 Population growth rate estimated from the 15 F1 hybrid clones obtained from the upscaled hybridisation experiment during a monitoring period of 10 days. Each parental clone combination was represented by five different F1 clones. Symbols represent mean values across clones. Error bars reflect variation among clones and equal twice the standard error of the mean. Different letters indicate significant differences according to Tukey post hoc tests ($p < 0.001$). *Brachionus elevatus*: 69_IIIB and 128_1B; *Brachionus calyciflorus* s.s.: 168_IIC and 69_IC

differed considerably in the production of dormant propagules ($\chi^2(2) = 341.7$, $p < 0.001$; Table S6); 69_IC♀ × 128_1B♂ clones were characterised by significantly higher frequencies of females with dormant propagules (Table 2) whereas we hardly observed any dormant propagules in the populations of parental clone combinations 168_IIC♀ × 69_IIIB♂ or 168_IIC♀ × 128_1B♂. Similarly, although all populations produced unfertilised sexual eggs, the frequencies of females with such eggs were considerably higher in the 69_IC♀ × 128_1B♂ than in the other populations ($\chi^2(2) = 100.7$, $p < 0.001$; Table S6). The fraction of abnormal dormant propagules equalled approximately 41%.

5 | DISCUSSION

Our study provides evidence for the existence of intrinsic postzygotic barriers between *B. calyciflorus* s.s. and *B. elevatus*. Intrinsic postzygotic barriers were found to mainly exist at the level of hybrid dormant propagule viability and the potential of F1 hybrids to reproduce sexually. The upscaled hybridisation experiment showed that abnormality occurred at a higher frequency in hybrid (60%) than in non-hybrid dormant propagules (36%). Abnormality of dormant propagules in brachionid rotifers has been shown to result in almost 100% mortality (García-Roger et al., 2005; Gilbert, 2010), which is confirmed by our study as well. Interestingly, we found no convincing evidence for a difference between hybrids and non-hybrids regarding the successful hatching of healthy-looking eggs, given that of the 35 dormant propagules that were successfully hatched and subsequently cultured as clonal line, almost half (15 clone lines) were hybrids. This suggests that hybrid zygotes of normally formed, healthy-looking dormant propagules have a similar potential of hatching and giving rise to viable, clonally reproducing populations as their non-hybrid counterparts. It should be noted, however, that non-hybrid dormant propagules were produced by selfing. Selfing may result in reduced fitness due to inbreeding depression (Birky, 1967; Tortajada et al., 2009) and we cannot a priori exclude that this resulted in increased abnormality rates and a reduced hatching success of non-hybrid propagules. This, nevertheless, renders our conclusion of a lower viability of hybrid compared to non-hybrid propagules more conservative. Furthermore, reduced hatching success of non-hybrid propagules may have prevented us from finding a difference for this trait between non-hybrid and hybrid propagules.

A second important postzygotic barrier was observed at the level of the ability of hybrids to produce sexual offspring with potentially important consequences for introgression-mediated gene flow between species. Clonal cultures of F1 hybrid clones displayed a large variation in the share of females with dormant propagules as well as in their exponential population growth rate. A substantial fraction of the variation in these variables was explained by parental clone combination. While all populations of F1 clones that originated from the parental clone combination 69_IC♀ × 128_1B♂ contained 6.6%–13.1% of females with unfertilised sexual eggs and proved able to produce normal dormant propagules (range: 10.3%–15.3%), the populations of the other F1 clones were characterised by a much lower production of unfertilised sexual eggs (range 0.3%–4.7%) and failed to produce dormant propagules with the exception of one (Table 2). In the latter populations, the low proportion of females with unfertilised sexual eggs suggests a low propensity for mixis induction, possibly as the result of a reduced signal recognition at the level of the sex inducing pheromone. This has undoubtedly contributed to low production rates of dormant propagules in the populations of these clones, although we cannot exclude that hybrid sterility has also played a role. Unfortunately, due to their near absence, we were unable to study the viability of

these dormant propagules. Partial or complete loss of the ability of hybrids to reproduce sexually has been reported in a wide range of organisms such as *Daphina* (Keller et al., 2007), grasshoppers (Finck & Ronacher, 2017), aphids (Delmotte et al., 2003), snails (Dillon et al., 2011), and lizards (Manríquez-Morán, Cruz, & Murphy, 2014). In line with our results, Schwenk et al. (2001) also showed strong variation in the production of ephippia, hatching, and juvenile survival rates between *Daphnia* hybrid clones, depending on the identity of their parental clones.

Our results also show that, depending on ancestry, specific hybrid clones (i.e. clones produced by 69_IC♀ × 128_1B♂) do have quite a high capacity to induce mixis and to produce males that are able to fertilise mictic females. Furthermore, fertilisation in this group of clones results in the production of dormant propagules of which the share of abnormal propagules is similar to what is observed among non-hybrid dormant propagules (e.g. 41%). Although we have no data on the hatching success of these propagules, the observed frequency of dormant propagule abnormality provides no evidence for enhanced hybrid sterility. It should be noted that we here documented the generation of F2 hybrid propagules produced through clonal selfing. As such, generalisation about the ability of F1 hybrid clones to produce backcrosses or genotypes with higher degrees of admixture should be done with care. However, such ability is plausible given that the postzygotic barriers related to sterility are likely to be most pronounced at the F1 level (Brill et al., 2016) and given that molecular analyses have suggested the existence of such hybrid classes in natural populations (Papakostas et al., 2016).

A large scale phylogeographic analysis combining data for mitochondrial and nuclear markers has shown a high degree of mitonuclear displacement in Dutch populations of *B. elevatus* and *B. calyciflorus* s.s. (Zhang, 2021). In all the investigated Dutch populations of *B. elevatus*, mtDNA proved to originate from *B. fernandoi*, whereas the mtDNA of the majority of *B. calyciflorus* s.s. populations originated from *B. elevatus*. A more detailed analysis of the phylogeographic origin of COI clades involved in these mitonuclear discordances revealed that current patterns of mitonuclear displacement in the Netherlands are likely to date from postglacial expansions and before and that introgression no longer affects the genetic structure of Dutch populations. Although these observations suggest a very effective reproductive isolation between Dutch *B. calyciflorus* s.s. and *B. elevatus* populations, very well in line with our experimental support for the existence of prezygotic (Zhang, 2021) and postzygotic barriers (current study), they are at odds with contemporary observations of hybrids and backcrosses in field populations (Papakostas et al., 2016; Zhang et al., 2019).

To solve this apparent paradox, it is important to consider that hybrids of cyclic parthenogens differ from hybrids of obligate sexually reproducing organisms in that they are able to circumvent barriers associated with sexual reproduction via clonal propagation (Delmotte et al., 2003). Despite important reproductive barriers, hybrids of cyclic parthenogens may be formed under specific circumstances and become locally successful if their clonal population growth is high relative to that of their parental species. For the species complex under consideration, there is indeed evidence that some hybrid clones have a higher potential for clonal population growth rate than parental species. In a laboratory evolution experiment, Lemmen et al. (2020) exposed populations to selection for fast population growth under different food quality conditions for a total of 36 days. Fourteen replicate populations initially consisted of the same set of 30 clones, of which 24 were *B. calyciflorus* s.s. and six were *B. calyciflorus* s.s. × *B. elevatus* hybrids. At the end of the experiment, two of the original hybrid clones were found to dominate eight of the populations. These results suggest that once formed, some hybrid clones potentially have a superior performance in clonal growth. The reason for this is likely that they invest less in sexual reproduction. Indeed, in a common garden experiment, Lemmen et al. (2020) observed that populations of the successful hybrid clones showed higher population growth rates and produced less males and dormant propagules than the populations of the non-hybrid *B. calyciflorus* s.s. clones. Our results suggest that such a trade-off exists also among hybrid genotypes. These observations are in line with other empirical studies conducted in cyclic parthenogens showing that sexual reproduction is costly and that a lower allocation of resources in sexual reproduction can facilitate faster clonal population growth (Zhang et al., 2019).

Once formed, hybrid clones with a low propensity for sexual reproduction may thus become relatively successful in the short time scale of single growing seasons, explaining why we are able to observe them in contemporary populations (Papakostas et al., 2016). However, their lower propensity for sexual induction is expected to reduce the probability of successful mating events with parental species or other hybrid classes. Furthermore, a reduced production of dormant propagules is likely to jeopardise their long-term persistence because it renders their populations more vulnerable to extirpation, especially in temperate regions where populations tend to mainly depend on recruitment from propagule banks during each spring for their re-establishment. Low production rates of dormant propagules will also reduce their capacity to disperse and colonise other water bodies. These limitations are expected to reduce the *lifespan* of hybrid clones and constrain their ability to realise lateral gene flow and eventually species reversal. This may also explain the absence of a signature of contemporary introgression on phylogeographic patterns of mitonuclear discordance (Zhang, 2021).

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AUTHORS' CONTRIBUTIONS

W.Z. and S.D. designed this study; W.Z. performed the experiment; W.Z. and S.D. carried out the statistical analyses and wrote the manuscript.

DATA AVAILABILITY STATEMENT

Data are present in the main document and Supporting information.

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SUPPORTING INFORMATION

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

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