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Transgenerational effect on sexual reproduction in rotifer populations in relation to the environmental predictability of their habitats

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Abstract

1. One way to cope with predictable environmental fluctuations that affect several generations of individuals is through nongenetic transgenerational effects. Ancestors match the phenotype of their descendants to the environment the latter are expected to experience. In facultatively sexual rotifer life cycles, sex is linked to the production of diapausing eggs, which enables survival between growing seasons. In some rotifer species, sexual reproduction is inhibited several generations after diapausing eggs hatch. We hypothesised that in ponds where the growing season length is predictable, rotifer clones reproduce asexually for more generations, allowing a fuller exploitation of the growing season and maximising production of diapausing eggs at the end of the season.
2. We tested this prediction by estimating the proportion of sexual females produced by several clones of the rotifer *Brachionus plicatilis* inhabiting eight ponds that vary in the predictability of the length of their growing season.
3. Rotifer clones from more predictable ponds were unresponsive to cues that induce sexual reproduction for more generations after leaving diapause than clones from unpredictable ponds. However, clones from more unpredictable ponds did respond to these cues from early generations, probably as a way to ensure the production of diapausing eggs against the risk of an unexpectedly early end to the growing season. Significant within-population clonal variation was observed in transgenerational responses.
4. Our results showed that the transgenerational inhibition of sexual reproduction observed in *B. plicatilis* evolved in clones from habitats where the length of the growing season (i.e., the period when populations are active in the water column) is more predictable on an interannual scale. Our findings also suggest that the longer the growing season, the more prolonged the inhibition effect (i.e., it affects a greater number of asexual generations).
5. This research shows the importance of understanding how organisms adapt to fluctuating environments through transgenerational strategies and establishes

the groundwork for future studies of the regulatory mechanisms underlying the transgenerational modulation of sex in rotifers.

KEYWORDS
across-generation plasticity, diapause, hydroperiod length, nongenetic effects, temporary ponds, zooplankton

1 | INTRODUCTION

Organisms are typically adapted to survive and reproduce within the range of environmental conditions experienced by their ancestors. Therefore, changes in the environment beyond this range reduce the fitness of an individual. Natural selection will favour the evolution of responses that allow individuals to minimise such reductions in fitness. The selection response will depend on the timescale at which the environment changes in relation to the lifespan of individuals and on the predictability of such change (Botero et al., 2015; Joschinski & Bonte, 2020). If the timescale of temporal variation is large enough, it is possible for a genetically polymorphic population to adaptively track environmental changes throughout generations by favouring particular genotypes under a regime of fluctuating selection. At shorter timescales, recent research has shown that a ubiquitous way to cope with time-varying environments affecting different generations is through nongenetic transgenerational effects, whereby ancestors can quickly match the offspring phenotype to changes in the environment (Bonduriansky et al., 2012, 2016; Chevin et al., 2010). For these transgenerational effects to evolve, environmental changes should be reliably anticipated or vary in a predictable fashion (e.g., Colicchio & Herman, 2020; Herman & Sultan, 2011; Leimar & McNamara, 2015; Lind et al., 2020; Yin et al., 2019). In contrast, if the environment varies unpredictably, then bet-hedging strategies may arise as optimal adaptive solutions (Bernhardt et al., 2020; Bonduriansky et al., 2012; Dey et al., 2016).

Nongenetic transgenerational effects refer to epigenetic processes of phenotypic expression control (i.e., those not accounted for by changes in the DNA sequence itself) that persist for three or more successive generations (Adrian-Kalchhauser et al., 2020; Baugh & Day, 2020). Although mediated by nongenetic processes of inheritance, transgenerational effects may underlie and influence many aspects of evolution (Agrawal et al., 1999; Day & Bonduriansky, 2011; Jablonka & Lamb, 2005; Pfennig, 2021). Interestingly, nongenetic transgenerational effects may be adaptive, maladaptive or neutral with regard to an individual's fitness (Agrawal et al., 1999; Burgess & Marshall, 2014; Donelson et al., 2018; Guillaume et al., 2016; Lane et al., 2015).

Among adaptive nongenetic transgenerational effects, the term transgenerational plasticity is employed for those cases in which the phenotype of an individual is affected by the phenotype or the environment of its ancestors (Bell & Hellmann, 2019; Mousseau & Fox, 1998). This is a kind of phenotypic plasticity occurring across generations that takes advantage of the temporal environmental correlation between successive generations and whose adaptive value is linked to the predictability of the environment (Engqvist & Reinhold, 2016; Proulx & Teotónio, 2017; Uller, 2008; Uller et al., 2013). Due to this correlation, parents have reliable information regarding the environments that offspring are most likely to encounter, so there is the possibility of modifying the offspring phenotype throughout the passage of generations to improve the match to the environment and enhance fitness (Agrawal et al., 1999; Dantzer et al., 2013; Donelan et al., 2020; Herman & Sultan, 2011; Mousseau & Dingle, 1991; Uller, 2008). From this perspective, in complex life cycles, it would be adaptive for parents to drive the phenotypes of their offspring depending on the environment that specific generations are expected to experience (i.e., the different generations of a given lineage experience only a fraction of an environmental cycle of change). This is the case for the so-called transgenerational interval timers, a phenomenon described in many insects (e.g., aphids: Campbell & Tregidga, 2006; Dixon, 1971, 1972; Lees, 1960, 1966; Margaritopoulos et al., 2002; Matsuda et al., 2017; Matsuda, Kanbe, et al., 2020; Matsuda, Numata, & Udaka, 2020; dipterans: Henrich & Denlinger, 1982; Zinovjeva, 1978; hymenopterans: Boivin, 1994; Reznik & Samartsev, 2015) and mites (Geispitz, 1968; Razumova, 1967), in which the expression of some life history traits appears to be inhibited for several generations by an endogenous timekeeper (working as a kind of hourglass biological clock measuring the duration of a time interval; Rensing et al., 2001). One of the best-documented instances of these interval timers at the physiological level is that of transgenerational inhibition of diapause in aphids, for which a refractory period of insensitivity to diapause-inducing conditions is observed for a number of post-diapause generations (Reznik & Voinovich, 2021). This effect is hypothesised to provide an adaptive mechanism for preventing an untimely diapause response for a period of time during which environmental conditions are usually optimal for growth (Henrich & Denlinger, 1982; Saunders, 2020).

Even though theoretical studies have predicted environmental scenarios leading to adaptive transgenerational plasticity (Bonduriansky et al., 2012; Hoyle & Ezard, 2012; Leimar & McNamara, 2015), it is only recently that experimental studies have put predictions to the test (e.g., Lind et al., 2020; Rescan et al., 2020; Shama, 2015; Walsh et al., 2016). However, the influence of environmental predictability on transgenerational plastic responses in nature still remains largely unexplored (Diaz et al., 2021; Groot et al., 2017; Lind et al., 2020; Munch et al., 2021; Yin et al., 2022). This is mainly due to the difficulty of finding both ecologically relevant traits and realistic environmental settings (Burton & Metcalfe, 2014; Donelson et al., 2018; Engqvist & Reinhold, 2016). In this research,

we address the key theoretical prediction of whether environmental predictability influences the magnitude of the response in a transgenerational effect, which could be based on a kind of interval timer. As we explain below, we take advantage of a well-studied habitat–population system consisting of rotifer populations inhabiting small brackish ponds in eastern Spain, which display a wide gradient in environmental predictability.

Rotifers of subclass Monogononta offer a suitable model system for the study of nongenetic transgenerational effects in animals, as shown by the huge variety of examples provided by Gilbert (2017). They have a complex life cycle that includes asexual and sexual reproduction and is conventionally called cyclical parthenogenesis because reproduction is mainly asexual—allowing population growth—with periods in which both asexual and sexual reproduction co-occur, the latter leading to diapausing egg production (i.e., diapause is linked to sex; Figure 1). The initiation of sexual reproduction in the life cycle depends upon a specific environmental stimulus (Gilbert, 1974, 2020), with population density being one common cue. However, there is some evidence of transgenerational regulation of the expression of sexual responses to these inducers. Specifically, in some species, the ability to initiate sexual reproduction is inhibited for several generations immediately after the hatching of the diapausing egg, even under strong stimuli that would normally induce sexual reproduction (Gilbert, 2002, 2003, 2017; Gilbert & Schröder, 2004; Seudre et al., 2019). This transgenerational effect, as suggested by Gilbert (2017), may be based on an interval timer analogous to those described in other organisms with complex life cycles (see above). Theoretical studies suggest that this effect may offer a possible advantage in populations with asynchronous diapausing egg hatching. Through this inhibitory effect, genotypes hatching late in the growing season could still grow when early-hatching genotypes have reached large numbers and with density great enough to induce sexual reproduction of the former (Gilbert, 2017, 2020; Serra et al., 2005).

Among monogonont rotifers, the eurihaline species *Brachionus plicatilis* has emerged as an ideal candidate for further investigation

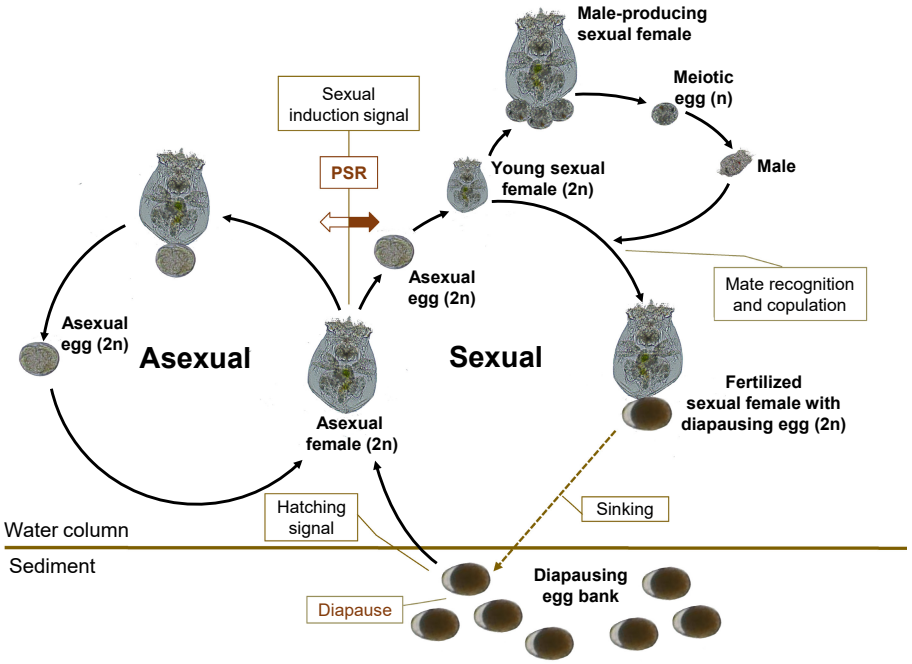

FIGURE 1 Typical life cycle of monogonont rotifers illustrated for the case of *Brachionus plicatilis*. At temperate latitudes, rotifer populations are typically temporary, with their growing season starting from the hatching of sexually produced diapausing eggs present in the sediment. The hatchlings are diploid asexual females that reproduce by ameiotic parthenogenesis for a number of generations. After this initial phase of clonal proliferation, sexual reproduction is triggered in the genus *Brachionus* in response to a threshold concentration in the medium of an infochemical produced by the rotifers themselves and is thus related to population density (Carmona et al., 1993; Snell et al., 2006; Stelzer & Snell, 2003, 2006). This causes parthenogenetic females to produce sexual females as a fraction of their offspring (proportion of sexual reproduction, PSR). The response of PSR to density can be described by a sigmoid function that shows a progression from null levels of PSR with low values of population density to later linear increase in a transition region from the population density threshold that induces sexual reproduction and then asymptotically approaches a maximum value of PSR in which sexual and asexual reproduction co-occur. Sexual females produce haploid eggs by meiosis, which, if not fertilised, develop into dwarf haploid males. If sexual females are inseminated while they are still young, their fertilised eggs develop into encysted dormant female embryos, which enter into diapause—becoming the so-called diapausing (or resting) eggs—and accumulate in the sediments, where they can resist adverse environmental conditions. After an obligate period of dormancy of variable length (Martínez-Ruiz & García-Roger, 2015), diapausing eggs hatch to restore clonal proliferation. If this occurs after an unsuitable period in the water column, a new growing season is initiated. In this manner, sexual reproduction is key for the production of diapausing eggs, representing the only way to survive unsuitable conditions between growing seasons (Serra et al., 2018). Rotifer microphotographs were acquired from I. Jezkova

of the adaptive value of transgenerational effects (Declerck & Papakostas, 2017; Serra et al., 2019). There are two features that make *B. plicatilis* particularly suitable for testing the hypothesis linking transgenerational effects to environmental predictability. On the one hand, the transgenerational inhibition of sexual reproduction across 24 clonal generations has been recently described in this species (Seudre et al., 2019). The effect is unequivocally ecologically significant, since it affects a life-history trait recognised as a key component of biological fitness in rotifers (i.e., the proportion of females in the offspring that are sexual). On the other hand, *B. plicatilis* is the rotifer species in which by far the most progress has been made in the study of the adaptive response to environmental fluctuation (Franch-Gras, García-Roger, Franch, et al., 2017; Tarazona et al., 2017) and in the characterisation of its habitats with respect to the predictable or unpredictable length of the growing season (Franch-Gras, García-Roger, Franch, et al., 2017; Franch-Gras, García-Roger, Serra, & Carmona, 2017). Note that the focus here is set on the predictability of interannual variation in the length of the growing season rather than in its average duration (whether it is in general short or long). We paid attention to predictability because the adaptive value of the transgenerational inhibition of sexual reproduction depends on the ability to anticipate the environment that the generations following diapausing–egg hatching will experience.

An adaptive hypothesis can be established by which rotifer populations from water bodies with a predictable length of the rotifer growing season would exhibit a transgenerational inhibitory effect on the sexual response to population density (the trigger for induction of sexual reproduction; see Figure 1) over several clonal generations to fully exploit the growing season maximise the production of diapausing eggs by the end of the season. In contrast, populations of rotifers living in very unpredictable ponds should display an early onset of sexual reproduction and avoid transgenerational effects to produce diapausing eggs as soon as possible in order to successfully survive an unexpectedly short growing season. Accordingly, in this study, we compared the transgenerational effects with respect to the proportion of sexual reproduction in *B. plicatilis* populations inhabiting ponds across a gradient of predictability. We used the data on the degree of environmental predictability of the natural habitats of a considerable number of *B. plicatilis* populations in Spain (Franch-Gras, García-Roger, Franch, et al., 2017; Franch-Gras, García-Roger, Serra, & Carmona, 2017) to test the hypothesis that the number of clonal generations during which the onset of sexual reproduction is inhibited will decrease with increasing environmental unpredictability.

2 | MATERIALS AND METHODS

2.1 | Experimental animals: Origin and maintenance of parental clones

A transgenerational effect on sex response to density was tested by estimating the proportion of sexual reproduction (PSR) exhibited by experimental clones (F-clones, from filial as explained below) of *B. plicatilis* at different generations from the hatching of the corresponding diapausing egg. These diapausing eggs were produced under controlled conditions in multiclonal laboratory populations generated by a mixture of clones (hereafter referred to as parental clones, P-clones). These P-clones were established directly from *B. plicatilis* natural populations inhabiting eight brackish ponds and lakes in eastern Spain. These habitats differ broadly in the degree of environmental predictability and hydroperiod length (Table 1; Franch-Gras, García-Roger, Serra, & Carmona, 2017). Briefly, environmental predictability for *B. plicatilis* populations focused on interannual variation in the length of the hydroperiod and was quantified by Franch-Gras, García-Roger, Serra, and Carmona (2017) from satellite data obtained over a 27-year period (1984–2011) and using Colwell's (1974) approach based on the presence or absence of water as a state variable. This predictability metric fits the case of *B. plicatilis* populations (i.e., takes into account its perception of environmental fluctuation) according to the biological information available for these ponds (details in Franch-Gras, García-Roger, Serra, & Carmona, 2017).

Parental clones were obtained by sampling the sediment egg bank, hatching the isolated diapausing eggs, and allowing parthenogenetic reproduction of the hatched stem females (details of the procedure are provided in Franch-Gras, García-Roger, Franch, et al., 2017). Therefore, P-clones were representative samples of the total genotype pool of each population, and since diapausing eggs are the product of sexual reproduction, each established clonal line represents a different genotype. In the present study, 10 P-clones per natural population were maintained individually in 13-ml stock cultures inside a walk-in chamber with controlled temperature (25°C) and constant illumination (photosynthetically active radiation: 35 μmol photons m^{-2} s^{-1}). Rotifer culture medium consisted of 12 g/L salinity water made with commercial sea salts (Instant Ocean®, Aquarium Systems) and enriched with f/2 medium (Guillard & Ryther, 1962) and contained the microalga *Tetraselmis suecica* (Kylin) Butcher 1959 (Microalgae Culture Collection of IATS-CSIC) as food (800,000 cells/ml; *c*. 105 mg C/L). Except where specified differently, the pre-experimental and experimental rotifer culture media and conditions were the same as those of the stock cultures (hereafter referred to as *standard conditions*). P-clone stock cultures were renewed on a weekly basis by transferring half of the culture to a new tube with fresh culture medium.

2.2 | Experimental design: Transgenerational effect on sexual reproduction

2.2.1 | Pre-experimental diapausing egg production by laboratory populations

Diapausing eggs were produced under controlled laboratory conditions to be used in the experiment described below. For each of the eight natural populations studied, three multiclonal laboratory

TABLE 1 Studied natural populations, named accordingly with the ponds they inhabit, and features of their habitats (adapted from Franch-Gras, García-Roger, Serra, & Carmona, 2017)

Natural populations	Abbreviation	Area of the pond (m^2)	Growing-season length predictability	Hydroperiod (fraction of the year flooded)
Pétrola	PET	1,190,000	1.00	1.00
Salobralejo	SAL	237,000	1.00	1.00
Atalaya de los Ojicos	ATA	47,000	0.75	0.93
Hoya Turnera	HTU	130	0.70	0.07
Hoya Yerba	HYB	1,060	0.34	0.23
Hoya del Monte	HMT	15,800	0.19	0.51
Hoya Chica	HYC	32,000	0.12	0.51
La Campana	CAM	29,000	0.11	0.63

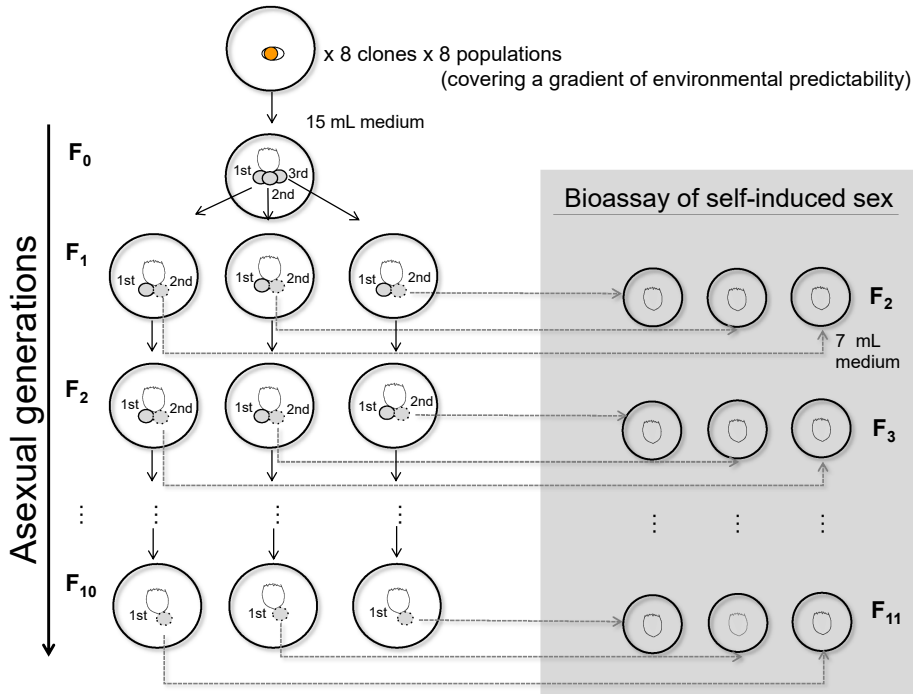

FIGURE 2 Diagram of the experimental procedure (propagation of generations and bioassay of self-induced sex) performed for each of the 62 experimental clones

populations—functioning as replicates—were generated by placing together five asexual females from each of the 10 P-clones (see above) in 400 ml of culture medium (initial density: 0.125 females/ml). These cultures were raised under standard conditions until they reached population densities allowing sexual reproduction and produced diapausing eggs (after approximately 15 days). Then, at least 150 diapausing egg-producing sexual females carrying one egg were isolated from each multiclonal laboratory population replicate. This procedure ensured that all the harvested eggs were very close in age. The diapausing eggs were stored in saline solution at 60 g/L in the dark at 4°C, conditions known to prevent hatching (García-Roger & Ortells, 2018), for at least 1 month to ensure the completion of the obligate period of dormancy (Hagiwara & Hino, 1989; Martínez-Ruiz & García-Roger, 2015) before experimental use.

2.2.2 | Foundation of experimental clones and propagation of generations

From the stored eggs previously harvested in the three replicates of each multiclonal lab population, at least 70 diapausing eggs per population were randomly selected and individually transferred to wells in polystyrene 96-well culture plates containing 0.1 ml of culture medium. Eggs were induced to hatch in saline solution at 6 g/L salinity under standard conditions (Martínez-Ruiz & García-Roger, 2015) and checked every 24 hr for hatchlings until eight stem females per population were obtained. Upon hatching, each stem female—constituting the F0 generation of each experimental clone—was cultured at low population density (<1 female/ml) in 60-mm diameter plastic Petri dishes containing 15 ml of culture medium of *T. suecica* at 250,000 cells/ml (*c.* 33 mg C/L). The first three daughters

(F1) served to establish three (replicate) lineages per clone in 15 ml of fresh culture medium. For each of these replicate lineages, subsequent generations, up to a maximum of 10 (F10), were followed by repeatedly initiating cultures with the first daughter from the preceding generation (Figure 2; Gilbert, 2002). When this initial female was sexual or died, the offspring was substituted by offspring of that same generation from another of the replicates of that clonal lineage, and was conservatively considered a pseudoreplicate. The second daughters from F1 to F9 (thus, F2 to F10 females) and the first daughter of F10 (F11 female) were used in the bioassay described below to estimate PSR. Due to logistical reasons, this procedure was not carried out simultaneously in all populations, but several experimental blocks were established, to which the diapausing eggs from the eight populations were randomly assigned.

2.2.3 | Bioassays of self-induced sexual reproduction

The sexual response (i.e., the proportion of sexual females generated among the offspring of an initial female in response to density) was estimated following the bioassay procedure of Gilbert (2002) with slight modifications. Neonate daughters obtained from generations F2 to F11 (see above) were individually isolated in 60-mm plastic Petri dishes containing 7 ml culture medium (Figure 2). The initial *T. suecica* concentration in the medium was set at ad libitum (250,000–500,000 cells/ml). An initial concentration of 250,000 cells/ml was used for several experimental blocks (including 35 F-clones), but the observation that a few clones might eventually deplete the food led to a precautionary concentration increase to 500,000 cells/ml for the rest of the experimental blocks (including 27 F-clones). For accurate control of food quality, the microalgae used in the bioassay culture medium were raised in a chemostat with a dilution rate of 0.295 per day. Reproduction and proliferation from the neonate females used in the bioassays were allowed under daily monitoring. After 4 days, sufficiently high densities of rotifers to induce sex occurred in the cultures. Exactly at that moment, rotifers were fixed with Lugol's solution (final concentration 4%). Females were counted under a stereomicroscope differentiating (1) gravid sexual and (2) gravid asexual females by the size and morphology of the oviposited eggs they carry (Carmona et al., 1995), and (3) non ovigerous females (juvenile plus post reproductive). From these counts, final female density (i.e., the total number of females per ml) and PSR (the proportion of ovigerous females that were sexual) were estimated for each bioassay culture. A total of 1,860 bioassays were performed as a result of combining 8 populations × 6–8 F-clones/population × 3 lineages/F-clone × 10 generations.

2.3 | Data analysis

Bioassays in which the final female density was lower than 6 or higher than 14 females/ml were discarded. These accounted for 857 out of 1,860 bioassays. The aims of this filtering were: (1) to ensure a sufficiently large sample size in the whole range of densities to obtain reliable estimates of the PSR; and (2) to select for a range of final density values representing a similar population density stimulus for the induction of sex in the bioassays. The range of density values considered is well above the threshold for inducing sexual reproduction in *B. plicatilis* (0.0002–2.28 females/ml; Carmona et al., 2009). Since F-clone replicate lineages were established from the first to third daughters of stem females, we discarded any possible effect of hatching order on PSR for each generation after diapausing egg hatching through correlation analysis (Figure S1). The numbers of sexual and asexual females in the sex self-induction bioassays were analysed by means of a generalised linear mixed model (GLMM). A binomial distribution of errors was assumed, and logit was used as the link function. Prior to model fitting, the existence of multicollinearity among predictors was checked. The GLMM included: (1) generation order, hydroperiod length, and predictability degree in the hydroperiod length of the origin locations as predictors, as well as their interactions; (2) female density in the bioassay cultures as a control covariate; and (3) sampled natural populations and F-clones within them as random effects (see the script *Colinas_et_al_Transgenerational effect.R* in Data S1). The significance of effects was tested through likelihood ratio tests between the full GLMM and reduced GLMMs obtained by single-term deletions using a threshold for $\alpha = 0.05$. Furthermore, the effects of early generations (resulting from grouping data of F2 and F3) versus late generations (F10 and F11) on the numbers of sexual and asexual females were studied by additional GLMM analyses for each population. These GLMMs considered F-clones as a random effect. Significance levels were corrected by the Bonferroni method for multiple tests. Finally, the Pearson correlation between PSR and final female density in the bioassays was computed. This relationship was studied to check whether the range of density values in the bioassays was far above the density threshold required for sex induction and for the PSR to have reached its maximum value (see caption in Figure 1). The relationship between PSR and female density was also explored by means of the calculation of slopes and intercepts, separately for the data subsets of early and late generations in each population. Again, significance levels were corrected using the Bonferroni method for multiple unidirectional tests.

All analyses were carried out using R 4.1.0 statistical software (R Core Team, 2021). GLMMs and likelihood ratio tests were run using the lme4 package (Bates et al., 2015), while correlation analyses were performed using the *cor.test* function from the *stats* package.

3 | RESULTS

Data from *c.* 1,000 bioassays were analysed to study the effects of generation order and predictability degree of the origin locations of derived experimental *B. plicatilis* clones on PSR. Our results showed a significant increase in PSR with generation order (Table 2), evidencing inhibition of sexual reproduction of F-clones in the first

few generations after leaving diapause and confirming the existence of a transgenerational effect in the phenotypic expression of this trait. The PSR varied with habitat predictability, showing higher levels of sex in the F-clones derived from the more unpredictable ponds (Table 2, Figure 3). A significant Generation × Predictability effect on PSR was also detected (Table 2). Interestingly, this interaction showed a positive coefficient, which was significantly different from 0 ($\beta = 0.7$, $z = 7.459$, $p < 0.001$). While PSR remained at high levels from the first generations in rotifer F-clones derived from ponds with higher environmental unpredictability, an inhibition in the sexual response was observed in the clones derived from the more predictable ponds, with PSR generally increasing gradually across generations (Figure 3). Our results also revealed the existence of genetic variability in PSR among F-clones within populations (i.e., significant effect of the clone factor; Table 2), but the response of the clones with respect to the transgenerational effect was generally consistent with the degree of predictability of their corresponding ponds (see Figure S2).

TABLE 2 Generalised linear mixed model analysis of the proportion of sexual reproduction (PSR) in the rotifer *Brachionus plicatilis*

Effect	*df*	*F*	*p*
Generation	1	50.97	<0.001
Predictability	1	9.74	0.002
Hydroperiod	1	6.69	0.010
Predictability × generation	1	56.49	<0.001
Hydroperiod × generation	1	52.24	<0.001
Hydroperiod × predictability	1	7.07	0.008
Generation × predictability × hydroperiod	1	47.61	<0.001
Population	1	3.09	0.079
Clone	1	797.27	<0.001

Note: Likelihood ratio tests (LRT) for the significance of fixed effects and their interactions. The effects of population and clone, treated as random factors, are also shown.

The comparison of PSR in the early (averaged F2 and F3) versus the late (averaged F10 and F11) generations assayed for each population showed the existence of significant differences in five of the eight rotifer populations studied (Figure 4). Figure 4 shows the PSR along the gradient of predictability of the studied ponds: notably, rotifer clones derived from most of the more predictable ponds (i.e., Salobralejo, Atalaya de los Ojicos, and Hoya Turnera) exhibited a significant increase in PSR from the first to the last generations assayed.

No significant relationship was found between PSR and total female density in the bioassay cultures in any of the studied populations after applying Bonferroni correction for multiple unidirectional tests (Figure 5). This observation held in the analysis by groups of generations (early or late). Thus, no significant slopes in the relationship between PSR and density were observed when considering the data from the early generations assayed in any population, although populations originating from more predictable ponds had on average much lower PSR values than those from ponds with higher environmental unpredictability for the same range of density values assayed. For the late generations, the lack of a relationship between PSR and density persisted, but in the case of the populations from the more predictable ponds, we observed higher PSR values than in the early generations (Table S1).

4 | DISCUSSION

Our study demonstrates a gradual increase in the sexual response to population density over 10 parthenogenetic generations following the hatching of diapausing eggs in 62 clones belonging to several

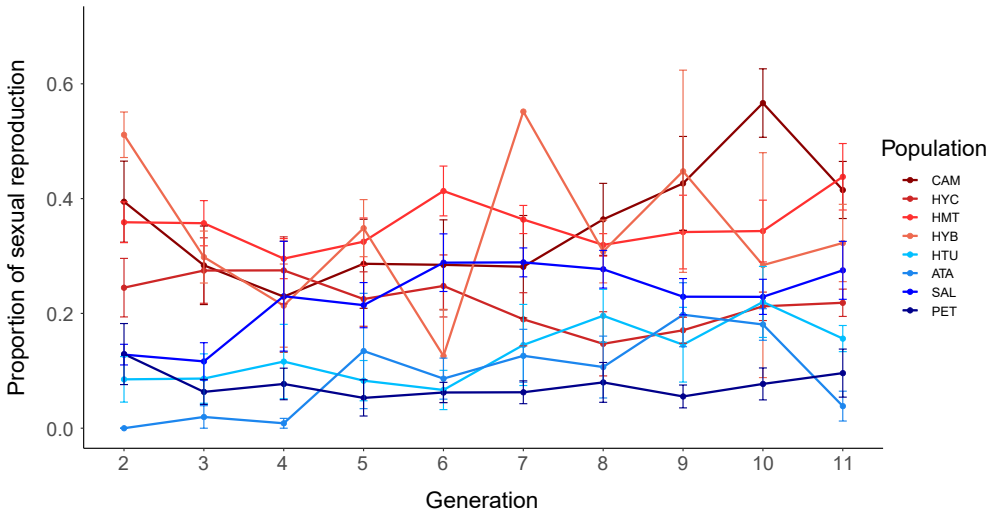

FIGURE 3 Proportion of sexual reproduction (PSR) in populations of the rotifer *B. plicatilis* across generations. The degree of predictability is coded with a gradient from darker red (rotifer populations inhabiting more unpredictable ponds) to darker blue (rotifer populations inhabiting more predictable habitats)

populations of *B. plicatilis*. This effect on the proportion of sexual reproduction after diapause confirms the results obtained recently by Seudre et al. (2019). These authors described for the first time in *B. plicatilis* (sensu stricto; after unambiguous taxonomic identification) that this trait is affected by nongenetic, maternal-mediated factors that may persist over several generations (however, see also Hagiwara et al., 2005; Hino & Hirano, 1977). Seudre et al. (2019) tested this effect on four clones during 18–24 generations and observed a gradual increase in the proportion of sexual reproduction over the first eight to 10 clonal generations up to a genotypically fixed maximum. These results hold based on previous observations reported first in the congeneric *Brachionus calyciflorus* (2002, 2003) and in *Brachionus manjavacas* (Kamizono et al., 2017), a sibling species of *B. plicatilis*, among other monogonont species (Gilbert, 1983; Schröder & Gilbert, 2004).

This kind of inhibitory mechanism of sexual reproduction has been proposed to evolve, permitting diapausing egg hatchlings to quickly colonise the habitat by promoting parthenogenetic growth (Gilbert, 2002, 2017; Schröder & Gilbert, 2004; Serra et al., 2005). It may prevent a genotype hatched in an environment already densely populated by conspecific genotypes from being induced to produce sexual offspring before it can increase its frequency in the population (Gilbert, 2002, 2003, 2017, 2020). Such a mechanism implies a transgenerational effect transmitted across multiple generations

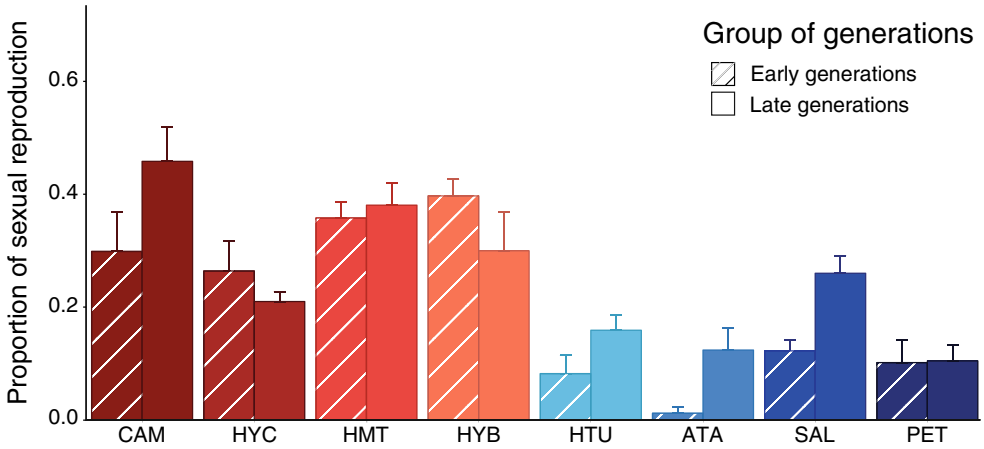

FIGURE 4 Proportion of sexual reproduction (PSR) in two groups of generations (*early*: F2 and F3; *late*: F10 and F11) in populations of the rotifer *Brachionus plicatilis*. Populations are ordered according to the predictability of their environments. Significance was tested by means of generalised linear mixed models with binomial responses and applying Bonferroni's correction (ns: $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$)

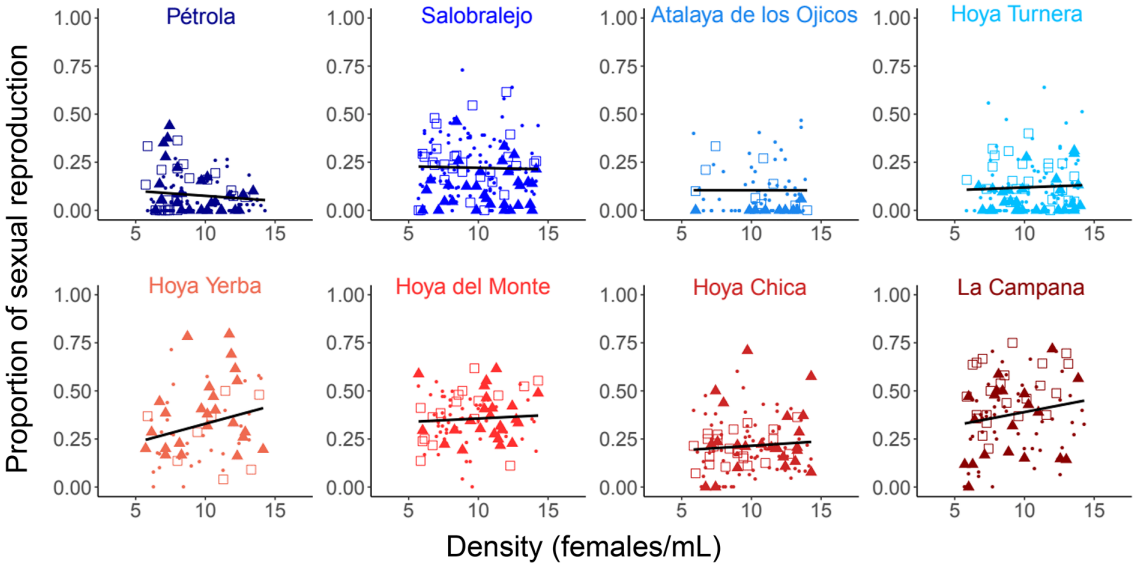

FIGURE 5 Effect of density on the proportion of sexual reproduction in *Brachionus plicatilis* populations (triangles: F2 and F3; squares: F10 and F11; circles: F4 to F9). Lines in each plot are linear regression fits. In all cases, the correlation (unidirectional test) was not significant after Bonferroni's correction ($p > 0.00625$)

by which early and late generations of the same genotype respond differently to similar environmental (i.e., population density) conditions. Furthermore, we conceive this transgenerational effect as an adaptive trait that modulates the expression of another trait across generations—in this case, the sexual response to the usual induction stimulus—that may be directly targeted by natural selection.

In this study, we provide the first empirical evidence of the adaptive value of this transgenerational effect in relation to the degree of growing-season length predictability experienced by *B. plicatilis* populations. In a previous study, Schröder and Gilbert (2004) tested for differences in the transgenerational effect of inhibition of sexual response in populations of other rotifer species (*Brachionus angularis*, *B. calyciflorus*, *Epiphanes senta*, and *Rhinoglena frontalis*) from a diversity of habitats. The results demonstrated considerable variability in the extent of this effect between populations within the same species, but no clear pattern was found with respect to habitat type. The study was limited to the comparison between the sexual response of the populations of some species from permanent and temporary habitats (assumed to be predictable and unpredictable) but was not strictly controlled (i.e., not all the studied species were present in both habitat types). Moreover, the environmental predictability of the studied habitats was not quantified. In this research, by studying clones from natural rotifer populations covering a gradient of environmental predictability (Franch-Gras, García-Roger, Franch, et al., 2017), we were able to provide support for the hypothesis that rotifer populations from more predictable water bodies exhibit an inhibition of sex in which they seem to be unresponsive to the stimuli inducing sexual reproduction over several clonal generations. It is worth noting that our bioassay design does not allow us to discriminate whether the inhibition of sexual reproduction was due to a loss of sensibility of the receptors to the infochemical that triggers sex or to a decrease in the production of this infochemical. In the latter case, late hatching clones could still respond to the infochemical produced by earlier hatching ones. Therefore, late hatching clones could start to reproduce sexually as soon as they hatch from the diapausing egg and the transgenerational effect would not work to prevent a premature investment in sexual reproduction (i.e., would not be adaptive). Contrary to this possibility, our results suggest that the trait does have adaptive value.

According to our predictions, rotifer clones derived from populations from more predictable ponds tended to exhibit lower PSR in the bioassays from early generations, with increasing PSR for the later generations. The increased PSR in the later generations of these clones did not change with the range of densities tested, suggesting that densities were not only high enough to induce sexual reproduction, but also allowed us to reach the maximum asymptotic value of the genotypically determined PSR (Montero-Pau & Serra, 2011). An exception to this trend was observed in the rotifer clones from Pétrola, one of the most predictable ponds in the study (Table 1), for which we observed a constant low PSR in the 10 generations tracked (and even in an additional five generations for three of the derived F-clones from this pond). We conjecture that rotifer clones from Pétrola could inhibit sex for more generations than those explored in this study. The fact that this pond exhibits long hydroperiods during which clones could continue to proliferate parthenogenetically supports this conjecture. By contrast, rotifer clones derived from ponds with more unpredictable growing seasons, such as La Campana, Hoya Chica, Hoya del Monte, and Hoya Yerba, showed high PSR values as early as in the first generation. A pattern of increased investment in sexual reproduction is expected in unpredictable environments, which would imply both (1) earlier initiation of sex (Franch-Gras, García-Roger, Franch, et al., 2017) and, once started, (2) higher PSR than in predictable environments. Nevertheless, hydroperiod length on its own may still exert effects on the variation in PSR across generations; a longer growing season could favour prolonged inhibition of sexual reproduction. This would explain the increase in PSR observed in the later generations assayed in clones from the La Campana population, eventually inhabiting a highly unpredictable pond but with a relatively long hydroperiod. We call for the need to consider hydroperiod length in relation to the generation times of the rotifers. Similarly, there is evidence that extremely short-lived habitats would select for no inhibition of sexual reproduction in rotifers. This is the case for the congeneric species *B. calyciflorus* in Laguna Fantasma (Argentina), where a high risk of predation occurring shortly after emergence from the diapausing egg may lead to early sexual reproduction (some diapausing eggs would be produced before active females are preyed upon) occurring at a low population density threshold (Gilbert & Diéguez, 2010). Another example of this effect occurs in *Hexarthra* sp. from Chihuahuan Desert ponds that could dry within days, where sexual females and diapausing eggs are observed 1–2 days after hatching (Schröder et al., 2007).

At the within-population level, the sexual response of the experimental clones with respect to the transgenerational effect was generally consistent with the degree of predictability of their corresponding pond. However, our results still show the existence of genetic variability in the PSR in *B. plicatilis* from the same population, as reported in this and other species (Schröder & Gilbert, 2004; Seudre et al., 2019). Genetic variation is necessary for the evolution of locally adapted traits. Therefore, our comparative analysis of habitat predictability suggests that both PSR and its transgenerational modulation are traits that may be under strong selection, so local adaptation of these traits would be expected to evolve. In this manner, remnant genetic variation implies some potential for future adaptation. This would explain how populations might be able to adapt from a set of founder clones from another locality. Regarding the mechanisms that maintain this genetic diversity, variation in hydroperiod length across growing seasons is a temporal environmental fluctuation that may work as a fluctuating selection alternatively favouring rotifer clones with diverging levels of transgenerational inhibition of the sex response to density. Furthermore, the existence of a diapausing egg bank leading to overlapping generations might help maintain this within-population genetic variation (Ellner & Hairston Jr., 1994; Ellner & Sasaki, 1996).

Our findings support the notion of a long-lasting adaptive non-genetic transgenerational effect occurring in rotifer populations.

According to Gilbert (2002), this nongenetic inhibition of sexual reproduction across generations meets the definition of adaptive transgenerational plasticity with the exception that, unlike most cases described (e.g., Bonduriansky, 2021), it is not induced by an environmental cue. Therefore, this plastic effect will be endogenously controlled and has been suggested to be triggered by the fertilisation of the oocytes of the sexual females, leading to diapausing egg formation and to the initiation of diapause (see Gilbert, 2002, 2017). In fact, there are other cases of transgenerational plasticity induced by fertilisation and concomitant diapause that affect morphological traits (Gilbert, 2002, 2017; Gilbert & Schröder, 2004). This mode of induction for the transgenerational effect in the sexual response in rotifers would be in line with the demonstration in species of aphids, dipterans, hymenopterans, and crustaceans that the experience of diapause by a female results in a decrease or even suppression of the incidence of diapause in her offspring for several postdiapause generations (e.g., Arbaciauskas, 2004; Bonnemaison, 1972; Brodel & Schaefers, 1979; Campbell & Tregidga, 2006; Dedryver et al., 2012; Dixon, 1971, 1972; Lees, 1960, 1966; Lushai et al., 1996; Margaritopoulos et al., 2002; Matsuda et al., 2017; Matsuda, Kanbe, et al., 2020; Matsuda, Numata, & Udaka, 2020; Ogawa & Miura, 2014; Voinovich & Reznik, 2017). This inhibitory effect triggered by the mother's diapause has also been described as transgenerational plasticity (Reznik & Samartsev, 2015; Tougeron et al., 2020), which is potentially regulated by an interval timer. Given the analogies, especially with aphids, a similar endogenous time keeping phenomenon could be at work in the case of the transgenerational effect observed in rotifers (Gilbert, 2017).

Although the mechanism for the inhibitory transgenerational effect in rotifers is still unknown, it may accommodate the so-called inherited gene regulation mechanisms (Adrian-Kalchhauser et al., 2020), which include cytoplasmic cellular components and non-DNA-bound factors—such as noncoding RNAs, nutrients, hormones and other metabolites—as well as genome-associated processes such as DNA methylation and histone modifications. In relation to the first type of mechanisms, Gilbert (2002, 2003, 2017) suggested that one possible mechanism in monogonont rotifers could be a kind of transmission of molecular information across generations producing changes in the phenotype expression of the sexual response to population density, but without changes in the genotype. This is a kind of nonnuclear mechanism involving a non–genome-associated signalling factor that would probably be released in response to fertilisation. Initially, the factor would be transferred through the yolk glands of diapausing egg-producing females to the cytoplasm of the diapausing embryos, and on to the stem females, leading to inhibition of sexual reproduction. Alternatively, the factor could be released as a consequence of undergoing diapause. Then, the factor would pass on from mothers to daughters through the eggs they produce, decreasing its concentration across generations. Thus, the inhibitory effect on sexual reproduction would manifest in each generation as long as the factor persisted. Gilbert (2017) suggested that such a factor may block the production of a steroid hormone involved in sexual reproduction, thus promoting parthenogenetic reproduction.

In relation to the second type of mechanism, the transgenerational modulation of the sexual response to density in rotifers could be accomplished by genome-associated inherited gene regulators, such as DNA methylation- or histone-mediated mechanisms (Jablonka & Raz, 2009), which are known to influence gene expression for more generations than cytoplasmic mechanisms (Anastasiadi et al., 2021). Interestingly, DNA methylation has been proposed to serve a role in the manifestation of interval timers in other organisms (Matsuda, Numata, & Udaka, 2020). To date, there is almost no information on DNA methyltransferase rotifer genes (Dnmts) and their methylation/demethylation patterns, but Kim et al. (2016) recently identified a Dnmt-2 homologue in the genome of *Brachionus koreanus*. Although elucidation of these mechanisms is beyond the scope of our study, this recent finding opens the possibility of studying whether changes in the expression of Dnmt-2 occur across generations in parallel with changes in both PSR and the expression of genes related to the induction of sexual reproduction in populations of rotifers whose habitats differ in the degree of growing-season length environmental predictability.

In summary, our observations provide evidence on how organisms adapt to fluctuating environments through transgenerational strategies. Many studies have demonstrated nongenetic changes in phenotypes spanning multiple generations in rotifers. The transgenerational response to sex-inducing stimuli is a well-documented effect in a broad range of rotifer species, but no previous study has explored the relationship between the degree of predictability in environmental fluctuations and the level of expression of this transgenerational effect. We have demonstrated that rotifer clones from more predictable environments have decreased sensitivity to stimuli inducing sexual reproduction in the early generations after diapause, while these same clones responded to population density after several generations. This transgenerational trait is adaptive in predictable environments since it allows proliferation despite the presence of conspecifics. In contrast, rotifer clones inhabiting unpredictable environments do not exhibit transgenerational inhibition of sex induction and can produce diapausing eggs as soon as possible to reduce their risk of extinction if the growing season is unexpectedly short. The underlying regulatory mechanisms for the transgenerational modulation of the sex response in rotifers are still very poorly understood, but our results may help in better designing the environmental settings in which possible mechanisms should be studied in the future.

AUTHOR CONTRIBUTIONS

Conceptualisation: M.J.C., E.M.G.-R. Developing methods: N.C., M.J.C., E.M.G.-R. Data analysis: N.C., M.S., M.J.C., E.M.G.-R. Preparation of figures and tables: N.C., M.J.C. Conducting the research, data interpretation, writing: N.C., M.S., M.J.C., E.M.G.-R.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial relationship that could be interpreted as a potential conflict of interest. There are no disputes regarding the ownership of the data presented in the paper, and all contributions have been attributed appropriately via co-authorship or acknowledgement, as appropriate to the situation.

DATA AVAILABILITY STATEMENT

Raw data are freely available on Zenodo.org (10.5281/zenodo.7701567).

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