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ROTIFERA XVI

Transgenerational expression profiles of a sex related and an epigenetic control gene in the rotifer *Brachionus plicatilis* in relation to environmental predictability

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Abstract A non-genetic transgenerational inhibitory effect on sexual reproduction has been demonstrated in *Brachionus plicatilis* in relation to environmental predictability. Indeed, clones of this species from more predictable environments do not respond to sex-inducing cues during several generations after leaving diapause. Notwithstanding, the molecular basis of this effect is still unknown. In this work, the expression level of genes related to the synthesis of sex hormones and to a potential epigenetic signalling mechanism were tracked along successive generations from diapausing eggs in clones of *B. plicatilis* populations inhabiting ponds with different level of environmental predictability. The selected genes were (1) the 17-β-dehydrogenase gene (*edh*), involved in the synthesis of 17-β-estradiol hormone in rotifers, and (2) the DNMT2 gene (*meth*), as a candidate epigenetic mechanism of control. According to expectations, results showed an increasing expression of *edh* across generations in clones from those the more predictable ponds. This finding provides a putative role of estradiol in the transgenerational effect. However, no differences were found in the *meth* gene neither across generations nor regarding the environmental predictability. Despite this, we point out alternatives for future research on the inherited gene regulation mechanism behind the transgenerational effect.

Keywords Transgenerational effect · Estradiol · DNA methylation · Rotifers · Environmental predictability

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Introduction

Cyclically parthenogenetic rotifers switch between reproductive modes in a density-dependent manner over their life cycle. Typically, rotifer females start reproducing asexually until sexual reproduction is induced in response to a threshold concentration of an infochemical produced by the rotifers themselves and released into the environment (Gilbert, 1963; Carmona et al., 1993; Stelzer & Snell, 2003, 2006; Snell et al., 2006). The result of sexual reproduction is the production of resistant diapausing eggs, which are crucial for the survival of temporary populations between growing seasons. Thus, rotifers produce diapausing eggs prior to the arrival of adverse conditions

and subsequently recolonize the habitat from the hatching of these eggs when a new growing season begins, thus, restarting the typical life cycle in the water column. Nevertheless, an inhibition effect has been reported for some populations of different species from genus *Brachionus, Epiphanes,* and *Rhinoglena* whereby the sexual response to density is markedly decreased or even absent for several generations after diapause (Gilbert, 1963, 2002, 2003; Schröder & Gilbert, 2004; Kamizono et al., 2017; Seudre et al., 2019; Colinas et al., 2023). This is a non-genetic transgenerational effect that manifests in delayed sexual reproduction, and it is proposed to be an adaptation that would allow rotifers to quickly colonize the habitat by promoting parthenogenetic growth (Schröder & Gilbert, 2004; Serra et al., 2005; Gilbert, 2017). This effect may prevent genotypes hatching in a densely populated environment (i.e. demographically dominated by conspecific genotypes) from being induced to reproduce sexually before reaching a large enough population density (Gilbert, 2002, 2003, 2017, 2020). Recently, the extent of this transgenerational effect was studied in relation to the degree of predictability in the pattern of variation of hydroperiod length across growing seasons in *Brachionus plicatilis* populations inhabiting a set of Mediterranean ponds (Colinas et al., 2023). Predictability in these ponds had been quantified by Franch-Gras et al. (2017a) and defined as the rotifer's ability to anticipate and adjust to future environmental conditions. Recently, Colinas et al. (2023) have found that *B. plicatilis* clones from populations inhabiting predictable ponds are unresponsive to sex-inducing cues for several generations after leaving diapause. However, clones from unpredictable ponds respond from early generations, which could serve as an adaptive strategy to ensure the production of diapausing eggs against an unexpected ending of the growing season.

Despite the above, the molecular mechanisms underlying the transgenerational effect on sexual reproduction found in rotifers are unknown. Gilbert (2002, 2003, 2017) has proposed that it may occur through an endogenous control mechanism involving some sort of maternally provided cytoplasmatic agent. A factor, yet to be determined, would be present in diapausing eggs inhibiting sexual reproduction in early generations, decreasing its concentration, and therefore, its effect across generations (Gilbert, 2002). It is worth mentioning that the concentration of this factor that would be transferred to the next generation would depend on the clutch size of each rotifer female, which could be a confounding factor.

Alternatively, the inhibition of sex in early generations after diapause could be mediated by any kind of transgenerational epigenetic inheritance mechanism (e.g. DNA methylation, histone modifications, or non-coding RNAs; Jablonka & Raz, 2009). DNA methylation is considered a common epigenetic signalling mechanism for the inhibition of gene expression (Bird & Wolffe, 1999; Bird, 2002) that may affect several generations (Angers et al., 2010) and that has been observed in many species (Adams, 1996; Field et al., 2004). Moreover, it was reported that DNA methylation is involved in the expression control of genes related to hormones in organisms with complex life cycles (e.g. the juvenile hormone in aphids; Walsh et al., 2010). DNA methylation is catalysed by a family of DNA methyltransferases (DNMTs) (Goll & Bestor, 2005). Of the three methyltransferases (DNMT1, DNMT2 and DNMT3) known in eukaryotes (Colot & Rossignol, 1999; Goll & Bestor, 2005), DNMT1 is typically involved in the maintenance of DNA methylation and DNMT3 in establishing new methylation marks (Bestor, 2000; Jaenisch & Bird, 2003; Goll & Bestor, 2005; Klose & Bird, 2006), whereas DNMT2 is involved in RNA methylation, specifically in tRNA (Goll et al., 2006). Notwithstanding, it has been recently reported that DNMT2 is also responsible of DNA methylation in some organisms (e.g. *Drosophila* sp.; Deshmukh et al. (2018)). To date, there is little information about methylation patterns in rotifers, but Kim et al. (2016) identified a single DNMT homologous to DNMT2 in *Brachionus koreanus*, and Franch-Gras et al. (2018) have also annotated a DNMT2 in the genome of *B. plicatilis*. Moreover, differences in the expression level of the DNMT2 gene have been found between diapausing eggs of *B. plicatilis* populations evolved experimentally under divergent regimes of environmental predictability (Tarazona et al., 2020). Thus, we hypothesize that this methyltransferase newly discovered in rotifers could play a role in the maintenance of the silencing of genes related to sexual reproduction.

Regardless of the inherited mechanism of gene regulation behind the transgenerational effect, one likely pathway over the control of sexual reproduction initiation in rotifers is through the synthesis of sex steroid hormones (oestrogens, androgens, and

progestins). Experimental studies have shown that exposure to these hormones increases sexual reproduction in rotifers (Gallardo et al., 1997, 1999, 2000a, b; Radix et al., 2002; Snell & DesRosiers, 2008). Interestingly, the exposure to 17-β-estradiol hormone produces an increase in the proportion of sexual reproduction in *B. plicatilis* (Gallardo et al., 1997), and the knockdown of the gene coding for the enzyme 17-β-dehydrogenase 12 (17-hydroxysteriod dehydrogenase 12), which is involved in the conversion of estrone in 17-β-estradiol, decreases sexual reproduction in *Brachionus manjavacas* (Snell, 2011). In this contribution, the expression level of genes related to the synthesis of sex hormones in rotifers and a potential epigenetic signalling mechanism were tracked along successive generations by quantifying mRNA from clones of *B. plicatilis* populations that originally inhabited ponds with different levels of environmental predictability. The genes were (i) the 17-β-dehydrogenase gene (hereafter, *edh*), involved in the synthesis of 17-β-estradiol hormone in rotifers, and (ii) the DNMT2 gene (hereafter, *meth*), as a candidate epigenetic mechanism of control. Our expectation is that the expression level of *edh* will increase along with generations in clones from the populations inhabiting the more predictable ponds, whereas it will remain constant in those clones from unpredictable ponds in agreement with previous results describing the transgenerational effect on the proportion of sexual reproduction in *B. plicatilis* (Colinas et al., 2023). Assuming that *meth* is a methylation maintenance gene for silencing the expression of other genes related to sexual reproduction, we hypothesize that its expression level will remain low in clones from populations inhabiting unpredictable ponds and that it will decrease its expression level across generations in the clones from the populations inhabiting the more predictable ponds.

Material and methods

Experimental design

The study of transcriptional changes across generations of *edh* and *meth* was performed on RNA samples obtained as part of a previous experiment described in Colinas et al. (2023), in which the transgenerational effect on the proportion of sexual reproduction in *B. plicatilis* populations was assessed. Briefly, in this experiment, populations inhabiting four saline ponds in Eastern Spain, which comprise a gradient spanning from low to highly predictable lengths of the growing season (Franch-Gras et al., 2017a) were tested (see Table 1 and Colinas et al., 2023, for further details). The environmental predictability for these *B. plicatilis* populations had been quantified by Franch-Gras et al. (2017a) from satellite data gathered over a 27 year period (1984–2011) using Collwell's (1974) index based on the presence or absence of water as state variable. This index considers the perception of environmental fluctuation by *B. plicatilis* individuals according to the biological information available for the studied ponds (details in Franch-Gras et al., 2017a). Several parental clonal lines (hereafter referred to as P-clones) were established by hatching diapausing eggs isolated from the sediment of each pond. Three laboratory multiclonal populations (functioning as replicates) were generated per wild population by placing together five females from each of 10 P-clones. Diapausing eggs from these multiclonal populations of approximately the same age and produced under controlled conditions were harvested and stored in saline solution (60 g l^{-1} Instant Ocean® Synthetic Sea Salts, Aquarium Systems) and in the dark at 4 °C for at least one month to ensure the completion of the obligate period of dormancy before experimental use (Hagiwara & Hino,

Table 1 Studied natural populations and features of the ponds where they inhabit: area (m^2), salinity (g/l), degree of environmental predictability, and hydroperiod length (adapted from Franch-Gras et al., 2017b)

Natural populations	Acronym	Pond area (m^2)	Salinity (g/l)	Estimated growing-season length predictability	Hydroperiod (fraction of the year flooded)
Atalaya de los Ojicos	ATA	47,000	17.53–54	0.75	0.93
Hoya Turnera	HTU	130	1.84–3.06	0.70	0.07
Hoya Yerba	HYB	1060	2.84–16.5	0.34	0.23
Hoya Chica	HYC	32,000	10.79	0.12	0.51

1989; Martínez-Ruiz & García-Roger, 2015). Experimental clones (hereafter referred to as F-clones) were haphazardly selected after hatching diapausing eggs obtained from the three replicates of each multiclonal population. Once 6–8 neonates per population had hatched, they were individually cultivated in 15 ml of saline water at 12 g l^{-1} with the microalgae *Tetraselmis suecica* as food source at 250,000 cells ml^{-1} (ca. 33 mg C l^{-1}). These females constituted the F0 generation of the F-clones. The first three daughters (F1) from each F0 female were individually transferred to new Petri dishes and cultured under the same conditions as the F0 females to establish three (replicate) lineages per clone. For each lineage within a clone, up to a maximum of nine (F9) subsequent generations were followed by repeatedly initiating cultures with the first daughter from the preceding generation (Fig. 1). The second daughters from the F1 to F9 generations, and the first daughter of F10, were used to perform a bioassay where the proportion of sexual females generated among the offspring of an initial female in response to density was estimated, so that data on the transgenerational effect on sex response are available (Colinas et al., 2023). The third daughters from F1, F3, F7, and F9 generations (thus, F2, F4, F8, and F10) served to obtain RNA samples under sex-inducing conditions like those in the bioassays and to estimate gene expression in the present research. For this purpose, neonate females were individually isolated in plastic Petri dishes (60 mm diameter) containing 7 mL of fresh culture medium (Fig. 1). The initial concentration of *T. suecica* in the culture medium was 500 000 cells ml^{-1}. Neonate females were allowed to grow and proliferate for 4 days, which is time enough for a high population density to be reached in cultures and for sexual reproduction to take place. Then, the cultures were filtered through 46.8 µm Millipore Nylon filters and washed with 12 g l^{-1} saline water to remove microalgal cells while retaining rotifers. Rotifers retained in the filters were fixed in liquid nitrogen and stored in Eppendorf tubes at – 80 °C until RNA extraction. A total of 360 cultures (4 wild populations × 6–8 clones/population × 3 lineages/clone × 4 generations) were processed in this way. Due to logistical reasons, the procedure was not carried out simultaneously in all the populations, but several experimental blocks were established, to which the diapausing eggs from the four populations were randomly assigned.

RNA extraction and qPCR

Total RNA was extracted from the 360 samples by resuspending and homogenizing the material retained in the filters in 400 µl of TRI Reagent® (Zymo Research). After that, RNA was purified using Direct-zol™-96 RNA (Zymo Research). RNA samples were retrotranscribed to cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen) and poli dT primers. The cDNA was stored at – 20 °C until expression quantification analysis. RNA concentration and purity of RNA, measured as the ratio of absorbance at 260 nm and 280 nm (A260/280), were evaluated spectrophotometrically using a NanoDrop (Thermo Scientific). Samples with a concentration lower than 10 ng μl^{-1} and A260/280 lower than 1.8 were discarded for expression quantification analyses. Primers for qPCR for the two genes of interest, *edh* and *meth,* were designed in exon/exon junction in order to avoid genomic DNA contamination. The sequences for these genes were obtained from the annotated *B. plicatilis* reference genome (NCBI: GCA_003710015.1; Franch-Gras et al., 2018) and blasted against NCBI database to search transcript reads (e.g. expressed sequence tags, transcriptomes shotgun assembly, and mRNA) to find the exon/intron boundaries. As no splicing information could be retrieved for reference genes previously used in rotifers, we analysed the stable and constitutive expression from available transcriptomic data (Tarazona et al., 2020) following Machado et al. (2020) in order to find putative housekeeping genes. The gene coding for the basic transcription factor 3 (*btf3*), which has been used as reference gene in other organisms (Bu et al., 2016), presented a high and homogenous expression level across different conditions and different splicing sites suitable for designing primers in exon/exon junctions and so was chosen as a reference for copy number standardization. Primers for *edh*, *meth,* and *btf3* were designed in Primer3Plus software with special settings for qPCR assays (Table 2). Specificity optimal amplification conditions of the primers were assessed through PCR using cDNA and genomic DNA, and amplicons were visualized by electrophoresis in agarose gels (30 min at 70 V, 2% agarose in SB buffer). Genomic DNA of the algae *T. suecica* was also assayed. In addition, PCR products were purified (QIAquick® PCR Purification Kit), quantified (Qubit™ 1X dsDNA HS Assay Kit), and sequenced

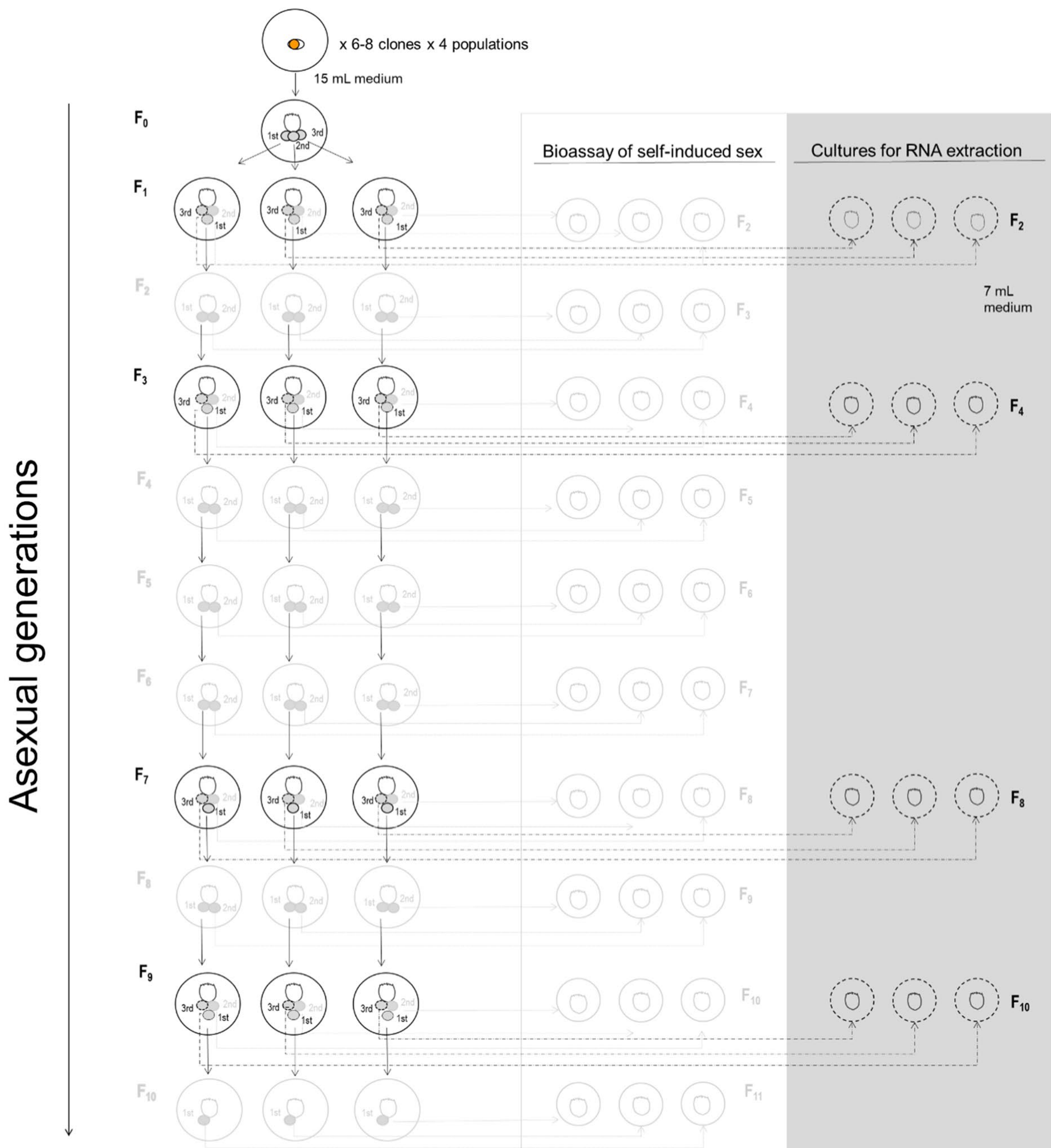

Fig. 1 Schematic diagram showing the experimental design performed for each *B. plicatilis* clone. RNA samples were obtained for 6–8 clones of each of the four studied populations as a part of a previous study carried out by Colinas et al. (2023)

by Sanger's sequencing in BMR Genomics (Padova, Italy).

The expression levels of the three genes of interest were quantified by qPCR using the CFX Connect Real-Time PCR thermocycler (Bio-Rad). The absolute quantification of each gene was carried out and the level of expression of the *edh* and *meth* genes (cDNA copy μl^{-1}) was normalized to the *btf3* cDNA copy μl^{-1} as previously described (Cucuzza et al., 2017). In detail, each qPCR analysis was performed

Table 2 Primer pairs used in the qPCR assays to detect transgenerational gene expression and qPCR-cycling conditions

Target gene	Primer name	Primer sequence (5′–3′)	Product size (bp)	Annealing temperature (°C)	No. of cycles
btf3	Btf3F	CGGCAGTCCAGAATACAGAAGA	105	62	32
	Btf3R	GTCTTGGAACACCTTTACCGC			
edh	EdhF	TCACAATTTACAGGGCTGGTTG	108	64	35
	EdhR	GCTTTTCGAATGGCTTTGGC			
meth	MethF	CCCGGAGAAATTATTGCTGCTG	107	66	35
	MethR	TTGATGCCTTCAATTGTCTTTTGT			

in a volume of 20 µl containing 2 µl of sample cDNA, 0.1 µl of each primer at 100 µM (and 10 µl of 2× SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). qPCR-cycling conditions are indicated in Table 2. Melt curve analysis was carried out from 60 to 95 °C with increments of 0.5 °C/5 s. All qPCR products were tested by electrophoresis (30 min at 80 V, 2% agarose gel in TBE buffer) to verify the size of the amplicons. The standard calibration curve for each gene was prepared following the approach published in Di Cesare et al. (2013). Briefly, PCR products, from the cDNA of each gene, were purified using a commercial kit (QIAquick PCR Purification Kit, Qiagen) and quantified as described above (expressed as ng μl^{-1}). Then, the purified amplicons were tenfold diluted in order to prepare the standard curve. In each qPCR assay, six standards (each one in duplicate), samples, and four No Template Controls (NTC) were included. The presence of PCR inhibitors in the samples was tested by the dilution method following Di Cesare et al. (2013), and no inhibition was observed. The reaction efficiency and R^2 for the assays were 93.82% ± 5.72% and 0.992 ± 0.007 (mean value of the three genes ± standard deviation), respectively. The ng μl^{-1} values obtained for both standards and samples were converted in gene copy μl^{-1} as described in Di Cesare et al. (2013): since 1 bp is equal to 1.095×10^{-12} ng, thus, knowing the concentration expressed in ng μl^{-1} and the size (bp) of each amplicon (shown in Table 2), the number of gene copy μl^{-1} was calculated. According to Bustin et al. (2009), the limits of quantification per each gene were 1700 copy μl^{-1} for *btf3*, 109 copy μl^{-1} for *edh*, and 158 copy μl^{-1} for *meth*. When the number of copies for a given sample was below the limit of quantification, the sample was considered as unquantifiable (Bustin et al., 2009) and removed from latter statistical analyses. The expression level of *edh* and *meth* was estimated in 76 and 47 samples, respectively.

Data analysis

The number of copies of *edh* and *meth* were normalized by the copy number of *btf3*, then transformed logarithmically and analysed by means of Generalized Linear Mixed Models (GLMMs). Gaussian distribution of errors and identity link function were used (Crawley, 2013). The formulation of GLMMs were identical for both genes and included generation order and predictability degree of the origin locations, as well as their interactions, as fixed-effect explanatory variables. F-clones were treated as levels of a random-effect factor. The significance of effects was individually tested through Likelihood Ratio Tests (LRTs) between the full GLMM and reduced GLMMs obtained after single term deletions using a threshold for α equal to 0.05. Non-significant terms were removed from the GLMMs to facilitate interpretation of effects. The validity of the resulting simplified GLMMs was tested using an approach based on the Akaike Information Criterion (AIC, Akaike, 1973). For the sake of better visualization in figures, generation order was conveniently grouped into early (F2 and F4) and late (F8 and F10) generations. This grouping did not affect data interpretation (for each gene, a GLMM considering this factor instead of generation order did not differ statistically from the GLMM described above, with negligible differences in AIC between models). Finally, by taking advantage of the data by Colinas et al. (2023), the relationship between the proportion of sexual reproduction (measured as the number of females in the offspring that are

sexual relative to the total number of ovigerous females) and the copy number of *edh* relative to *btf3* was studied for the same group of populations and clones using Pearson's correlation analysis. All analyses were performed using R 4.1.0 statistical software (R Core Team, 2021). GLMMs and LRTs were run using the "lme4" package (Bates et al., 2015) and correlation was performed using the *cor.test* function from the "stats" package.

Results

Results showed decreasing expression levels of *edh* with increasing environmental predictability of the origin ponds of the clones and populations studied (Fig. 2A), this effect was statistically significant (Table 3). Interestingly, results also showed an increase in the expression level of *edh* when comparing early and late generations in the populations of

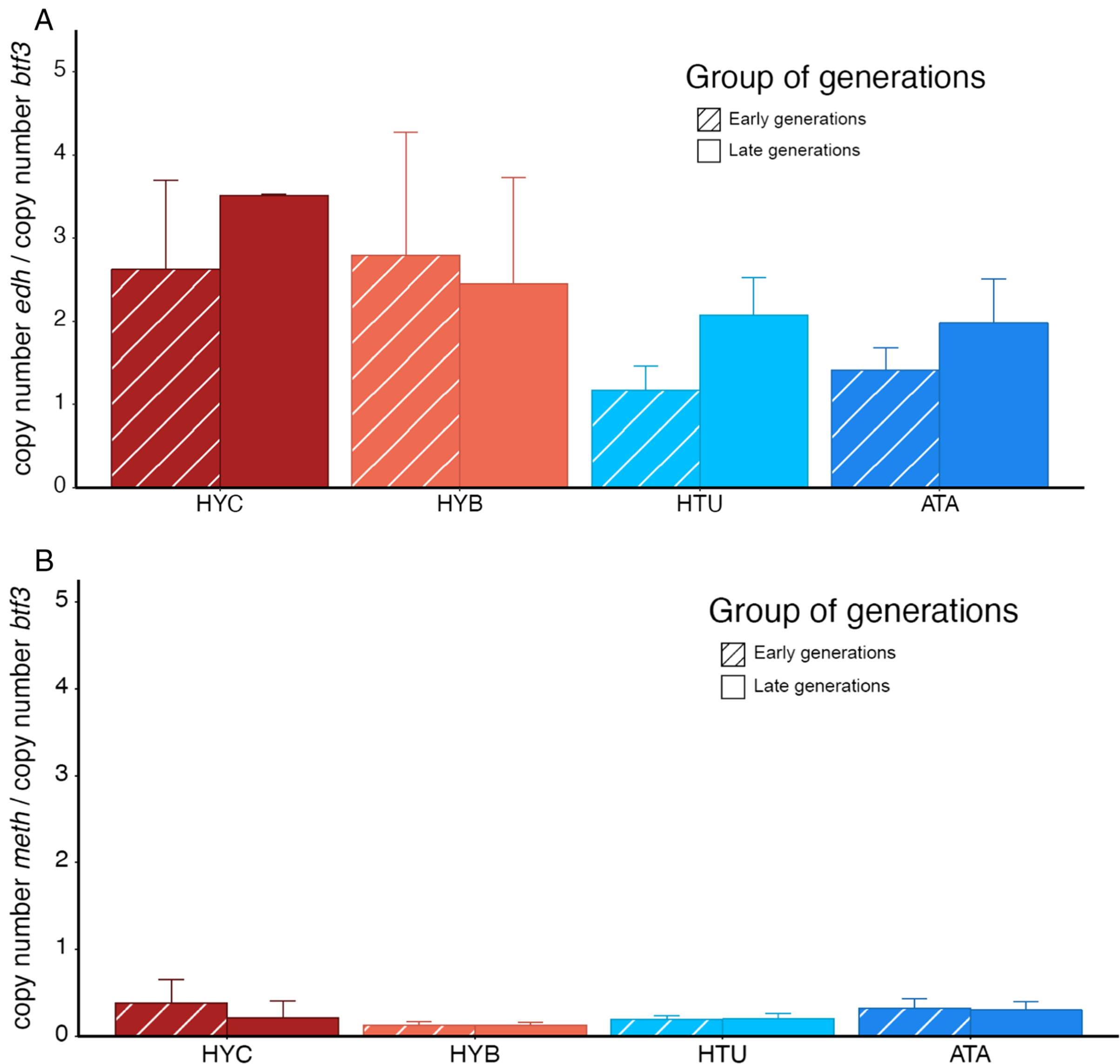

Fig. 2 Comparison of the expression level of **A** the *edh* gene and **B** the *meth* gene between two groups of generations ("early": F2 and F4; "late": F8 and F10) in populations of *B. plicatilis* from four ponds (HYC, HYB, HTU and ATA). Environmental predictability is coded using a colour gradient from darkest red (more unpredictable ponds) to darkest blue (more predictable ponds)

Table 3 Effect of generation order, habitat predictability and their interaction on the logarithmic copy number in the four studied populations of rotifer *Brachionus plicatilis* for *edh* and *meth* genes

Effect	*df*	*edh*		*meth*	
		F	*P*	*F*	*P*
Generation	1	2.460	0.117	3.42	0.064
Predictability	1	7.100	0.008	0.011	0.916
Generation × predictability	1	4.203	0.040	1.003	0.317

the most predictable ponds (Fig. 2A), the interaction effect between generation order and predictability was significant too (Table 3). Congruently, the coefficient for the interaction term was positive and significantly different from 0 ($\beta = 0.4$, $t = 2.022$, $P < 0.02$). No differences between early and late generations in expression level of *edh* were found in the populations from the most unpredictable ponds. The expression level of *meth* was much lower than that of *edh* (Fig. 2B) and none of the effects tested by GLMM were significant (Table 3). No differences were found in the transgenerational variation of gene expression of the clones within each population for either *edh* (LRT $\chi^2 = 1.929$, $df = 2$, $P = 0.381$) or *meth* (LRT $\chi^2 = 3.420$, $df = 2$, $P = 0.181$). The relationship between the expression level of *edh* and the proportion of sexual reproduction measured in several clones from the populations studied) was positive and significant (Fig. 3; $r = 0.62$, $P = 0.019$).

Discussion

Many studies have shown the inhibition of sexual reproduction across the early generations following diapause in rotifers (Hino & Hirano, 1977; Gilbert, 2002, 2003; Schröder & Gilbert, 2004; Kamizono et al., 2017; Seudre et al., 2019). In a recent experiment in *B. plicatilis,* the contribution of environmental predictability to this transgenerational effect has been demonstrated and its importance in the adaptation of rotifers to fluctuating environments highlighted (Colinas et al., 2023). Nevertheless,

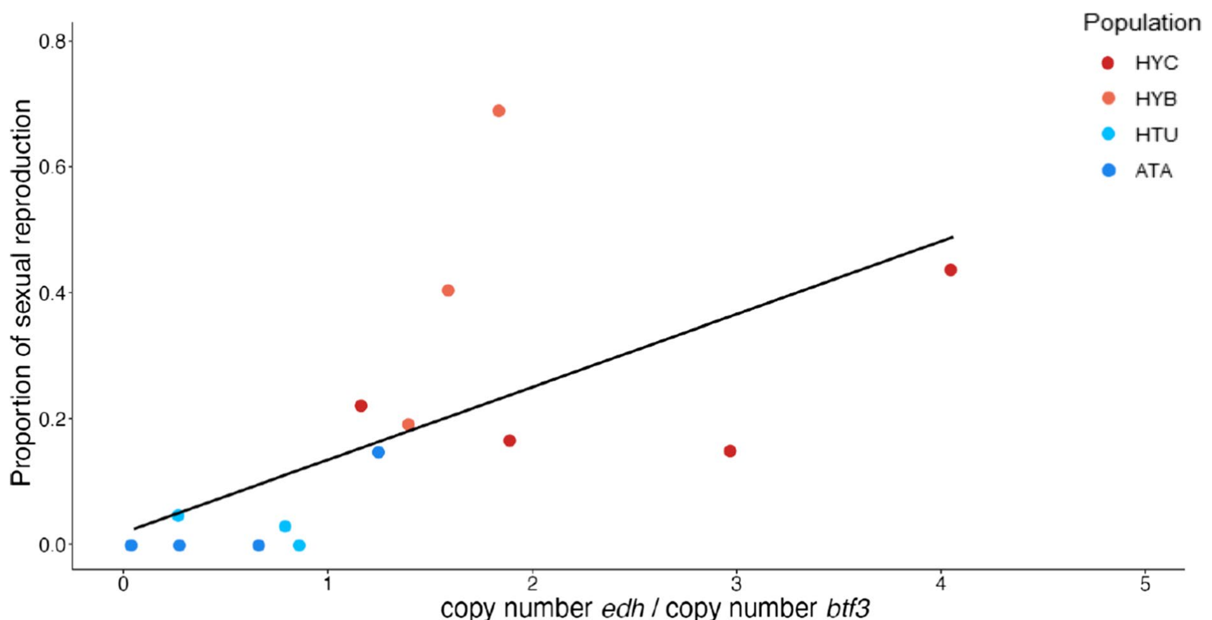

Fig. 3 Relationship between the proportion of sexual reproduction (data from Colinas et al., 2023) and *edh* copy number from the same set of *B. plicatilis* clones from each studied population. Environmental predictability is coded using a colour gradient from darkest red (rotifer populations inhabiting more unpredictable ponds, HYB and HYC) to darkest blue (rotifer populations inhabiting more predictable ponds, ATA and HTU). Trend line represents the linear regression fitted to the data

little was known about the underlying molecular mechanisms. Therefore, the main aim of the present research was addressing the expression profile of two genes that could be involved in the non-genetic inheritance of this transgenerational effect. In line with initial expectations, the results showed a significant increase in expression level of *edh* across generations in the clones coming from rotifer populations inhabiting predictable environments, whereas no differences between early and late generations were observed in the clones coming from populations inhabiting unpredictable ponds. Since *edh* encodes for 17-β-dehydrogenase 12 enzyme, which is required to catalyse the interconversion of estrogenic hormones estrone and 17-β-estradiol (Mindnich et al., 2004; Moeller & Adamski, 2009), the low expression of the gene would be expected to result in a reduced level of estradiol. Sex steroids are involved in reproductive processes in aquatic invertebrates (Khöler et al., 2007; Lafont & Mathieu, 2007; Miglioli et al., 2021) and there is experimental evidence supporting that estradiol signalling may be an important mechanism regulating rotifer reproduction in different ways (Snell, 2011; Jones et al., 2017). First, genes encoding for key enzymes required in steroid biosynthesis, including 17-β-dehydrogenase 12 have been identified (Snell, 2011) in *B. plicatilis* transcriptomes (Suga et al., 2007; Denekamp et al., 2009). Second, the selective silencing of *edh* gene expression decreased sexual reproduction in *B. manjavacas* (Snell, 2011). Third, genes encoding for estrogen-like receptors have been reported in several *Brachionus* species including *B. plicatilis* (Kim et al., 2017) and a ligand-activated estrogen-like receptor was shown to bind 17-β-estradiol and regulate reproduction in *B. manjavacas* (Jones et al., 2017). Fourth, exposure to estradiol caused a twofold increase in the proportion of sexual reproduction in *B. plicatilis* (Gallardo et al., 1997). Despite endocrine-like bioregulation in rotifers needs to be more clearly elucidated, the presumed regulation of the production of sexual females by estradiol will be in accordance with our finding that the proportion of sexual reproduction measured by Colinas et al. (2023) is significantly and positively correlated with *edh* expression level quantified in the present research for the same set of clones. Moreover, the involvement of 17-β-estradiol provides a putative estrogenic-mediated pathway for the transgenerational effect observed in *B. plicatilis* inhabiting predictable ponds (Colinas et al., 2023). Low levels of 17-β-dehydrogenase 12 expression, and therefore of 17-β-estradiol, could lead to the low proportions of sexual reproduction in response to sex-inducing signals found in the first generations after diapausing egg hatching. Accordingly, the expression levels of *edh* were higher in populations from unpredictable ponds than in those from predictable ones coinciding with the direct relation between the level of environmental unpredictability and the investment in sexual reproduction reported in previous studies (Franch-Gras et al., 2017b, 2019; Tarazona et al., 2017). A comment regarding our estimate of the degree of environmental unpredictability in the ponds from which the studied populations originate is pertinent here. Predictability was estimated on the across-year variation in the length of the hydroperiod of each of the studied pond, which was used as a proxy for the length of the growing season for *B. plicatilis* Franch-Gras et al. 2017a, b*;* Colinas et al., 2023). Of course, other environmental factors could vary in a roughly predictable fashion and affect the actual length of the growing season. Among these factors, one may cite extremes of environmental conditions (e.g. salinity), food availability, or the presence of antagonists such as competitors or predators, whose timing in the growing season should be reliably anticipated and matched by the transgenerational effect.

Once established how the changes in the expression level of *edh* might be related to the transgenerational inhibition of sexual reproduction in *B. plicatilis*, the question remains about the epigenetic control mechanisms of gene expression behind the transgenerational effect. In this contribution we proposed that DNA methylation could play a role, since it is an epigenetic mechanism typically linked to gene silencing, whose effects can extend over a considerable number of generations. Thus, we have focused on a homologue of DNMT2 methyltransferase found in rotifers (Kim et al., 2016; Franch-Gras et al., 2018), here named *meth*, whose expression could be related to the transgenerational effect by being involved in housekeeping DNA methylation. However, we did not find changes in the expression level of *meth* across generations, since its expression was similar between early and late generations after diapause. In fact, the expression level of *meth* was generally low, both in rotifer populations from unpredictable and predictable ponds.

Nonetheless, an exploration of gene expression data in *B. plicatilis* diapausing eggs produced under divergent selective environments regarding predictability in the length of the growing season (predictable vs unpredictable; Tarazona et al., 2020) has shown a higher expression of *meth* in diapausing eggs from predictable environments ($F = 63.134$, $df = 1$, $P = 0.016$). This suggests the possibility that there are differences in the starting methylation level between rotifer stem females from predictable and unpredictable environments and that perhaps a passive demethylation process allows reversing the silencing of genes related to sexual reproduction as generations pass. Of course, this is a matter for further investigation as we cannot rule out the possibility that other epigenetic mechanisms—e.g. histones or small RNAs—could be involved in the expression control of particular genes in rotifers (e.g. Lee et al., 2020).

Our results attempted to shed light on the molecular mechanisms implied in the non-genetic transgenerational effect of inhibition of sexual reproduction in *B. plicatilis*. We have documented a possible role of the 17-*β*-estradiol hormone mediated by the expression of *edh* encoding for the 17-*β*-hydroxysteriod dehydrogenase 12 in *B. plicatilis*. The increase of *edh* expression across generations in populations from predictable environments correlates with a high investment in sexual reproduction in late generations in these environments, as found in other studies. This means that at the molecular level we can track the timing of sexual reproduction and the investment in it by quantifying 17-*β*-estradiol levels or the increase in *edh* gene expression. Despite the above, it remains to be found a mechanism that explains the transgenerational effect. Our results do not support the conjecture about a housekeeping methylation for the silencing of genes related to sexual reproduction, including *edh*. Whether the mechanism behind the transgenerational effect inhibiting sexual reproduction in the early generation after diapause is a spontaneous demethylation process—compatible with our finding of differences in the levels of methyltransferase activity in diapause embryos produced in divergent regimes of environmental predictability—or whether it is mediated by histones, microRNAs, or by other cytoplasmatic factors that affect gene expression yet to be determined, will be the subject of future research.

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Author contributions Conceptualization: EMG-R and MJC; developing methodology: NC and JM-P. Conducting the research: all authors. Formal analysis: NC and EMG-R; visualization: NC, EMG-R, JM-P and MJC. Project administration: EMG-R. First-draft elaboration: NC. Writing: MG-R, JM-P, MJC and NC; review and editing: all the authors. Funding acquisition: MJC. All authors read and approved the final manuscript.

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Data availability The authors confirm that the data supporting the findings of this research are available within the article and/or its supplementary materials.

Declarations

Competing interests 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.

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