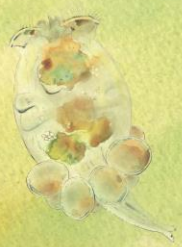

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PhD Thesis
December 2024

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Doctoral Programme in Biodiversity and Evolutionary Biology

Supervised by:
Dr Eduardo García Roger
Dr María José Carmona Navarro

Institut Cavanilles de Biodiversitat i Biologia Evolutiva

Laboratorio de Ecología Evolutiva

Transgenerational effects in an ecological context: sexual reproduction and environmental predictability in the rotifer *Brachionus plicatilis*

Efectos transgeneracionales en un contexto ecológico: reproducción sexual y predecibilidad ambiental en el rotífero Brachionus plicatilis

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PhD Thesis/*Tesis Doctoral*

Noemi Colinas Vallejo

December 2024

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CERTIFY that **Mrs. Noemi Colinas Vallejo** has carried out under our direction the research work included in this doctoral thesis, entitled: "**Transgenerational effects in an ecological context: sexual reproduction and environmental predictability in the rotifer *Brachionus plicatilis***", to obtain the degree of PhD at the University of Valencia. And for the record, in compliance with current legislation, we issue this certificate in Paterna, December 2024.

CERTIFICAN *que* ***Doña Noemi Colinas Vallejo*** *ha realizado bajo nuestra dirección el trabajo de investigación incluido en esta tesis doctoral, que lleva por título* ***"Efectos transgeneracionales en un contexto ecológico: reproducción sexual y predecibilidad ambiental en el rotífero Brachionus plicatilis"*** *para optar al grado de Doctora por la Universitat de València. Y para que conste, en cumplimiento de la legislación vigente, expedimos el presente certificado en Paterna, diciembre 2024.*

Eduardo Moisés García Roger María José Carmona Navarro

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Summary in English

Introduction

Habitats in which organisms live present numerous abiotic and biotic factors that can vary temporally and/or spatially. These environmental fluctuations are ubiquitous and diverse in nature. To persist in these variable environments, organisms must have strategies, including physiological, morphological, biochemical, behavioural and life history traits that mediate responses to fluctuations. Understanding these strategies requires exploration of the patterns of environmental variability, the traits that mediate responses throughout the life cycle, and the consequences of those responses affecting fitness, driving adaptation, shaping population dynamics and altering evolutionary trajectories. Hence, from both a fundamental and applied point of view, these are considered central questions in evolutionary ecology.

This thesis deals with time-varying habitats and therefore with the responses to temporal heterogeneity. The mode of response that is expected to evolve in such environments depends on the timescale of variation relative to the organism and the predictability of that variation. For organisms living in temporally variable but predictable environments, it is phenotypic plasticity what is expected to evolve as adaptive strategy. Plastic responses can be reversible or irreversible, depending on whether the phenotypic change reverts to its original condition or not, and may

involve one or more generations. This thesis studies a case where an individual's phenotype is under the control of prior generations of the same genetic lineage. Adaptive nongenetic transgenerational effects encompass transgenerational plasticity, a form of phenotypic plasticity operating across generations whereby the phenotype of an individual is affected by the phenotype or the environment of its ancestors. According to theory, the adaptive value of transgenerational plasticity is linked to the existence of temporal environmental heterogeneity and the ability of the parental generation to predict the environmental conditions that the successive generations will experience. Theoretical and experimental studies suggest that a high environmental correlation across generations enhances predictability, which can drive the selection for adaptive transgenerational effects.

Organisms living in time-varying environments are challenged to schedule the timing of the different stages and events of their life cycle with the occurrence of favourable conditions. This scheduling is even more relevant for organisms with complex life cycles (i.e., those in which organisms undergo several stages that differ in form and/or function), especially those entering a resistance phase or switching between modes of reproduction. Selection operates to maximize fitness throughout the life cycle, integrating the entire reproductive performance of a genotype, whether it is an individual or a clonal linage (i.e., a set of organisms derived from a single parent by asexual reproduction, and which are

genetically identical except by mutation), and whereby the life history traits are the major phenotypic components of fitness. Therefore, in complex life cycles where several asexual generations are interspersed between sexual reproductive events, the different generations of a given lineage experience only a fraction of an environmental cycle of change, and it would be adaptive for ancestors to influence the phenotypes of their asexual offspring depending on the environment that specific generations are expected to encounter. In such cases, transgenerational effects link the phenotype, or the environment experienced by ancestors to the life history traits expressed in the offspring. A striking instance of this are the so-called transgenerational interval timers, a phenomenon described in many insects and mites in which the expression of some life history traits appears to be inhibited for several generations in the offspring of females that had undergone diapause. This transgenerational plastic inhibitory effect is thought to be based on an endogenous timekeeper working as a kind of hourglass biological clock measuring the duration of a time interval.

Diapause is a common life history trait among invertebrates with complex life cycles. It is a form of internally controlled dormancy that encompasses a suspended period of development and is considered an adaptation to survive unsuitable conditions in time-varying environments. The diapause stage is, therefore, an adaptive and plastic phenotype essential for the survival of a genotype and it can occur at any stage of the life cycle

such as eggs, embryos, larvae, pupa or adults, but each taxa exhibits diapause in a specific stage. Diapause can be induced prior to the onset of adverse conditions (e.g., extreme values of temperature, drought, darkness, presence of competitors and predators, etc.) or in direct response to them before becoming critical. This, in the case of facultative diapause, requires individuals to recognize signals (i.e., temperature, food concentration, photoperiod, population density, etc.) that indicate that the environment has started to deteriorate and thus initiate diapause by means of endogenous control mechanisms. Several transgenerational effects on diapause induction have been reported, mainly in insects, crustaceans and rotifers.

Rotifers of the subclass Monogononta are small aquatic invertebrates are mostly found in the plankton of continental water bodies. They present a complex life cycle that includes asexual and sexual reproduction. This life cycle is conventionally called cyclical parthenogenesis because reproduction is mainly asexual with episodes of concomitant sexual reproduction that leads to diapausing egg production. At temperate latitudes, rotifer populations are typically temporary, with their growing season (i.e., the period of time in which a rotifer population is active in the water column) starting from the hatching of diapausing eggs present in the sediment. The hatchlings of these eggs are diploid asexual (amictic) females that reproduce by ameiotic parthenogenesis for several generations. After this initial phase of clonal proliferation, the initiation

of sexual reproduction depends upon a specific environmental stimulus, with population density being one common cue. In the genus *Brachionus,* and in other species, sexual reproduction is density dependent, triggered in response to a threshold concentration in the medium of an infochemical produced by the rotifers themselves. The infochemical triggers asexual females to produce sexual (mictic) daughters as a fraction of their offspring. Sexual and asexual females are morphologically indistinguishable, but they can be differentiated by the size and morphology of the oviposited eggs they carry. Sexual females produce haploid eggs by meiosis, which, if not fertilised, develop into haploid progenetic dwarf males. Female insemination is possible for only a few hours after birth, quite a bit earlier than maturity. A male can inseminate more than one female, but a single insemination is enough to fertilize all the eggs a sexual female will produce. If sexual females are inseminated while they are still young, their fertilised eggs develop into encysted dormant female embryos, which enter diapause becoming the so-called diapausing (or resting) eggs. These eggs settle in the sediments where they can resist adverse environmental conditions (e.g., desiccation, extreme salinity and temperature, competitors, predators and parasites) remaining viable for decades, even centuries. After an obligate period of dormancy of variable length, and when suitable conditions resume in the water column, a fraction of the diapausing eggs hatches into asexual females to restore the active population and a new growing season

begins. Since some of these eggs do not hatch in the following favourable season after settlement and remain in the sediments for longer periods, they form a diapausing egg bank. In this way, sexual reproduction is key to producing diapausing eggs, representing the only way to survive unsuitable conditions between growing seasons.

Several studies have proven an inhibition in the ability to initiate sexual reproduction –and therefore to produce diapausing eggs– in response to population density in several generations after leaving diapause in rotifers. The nongenetic inhibition of sexual reproduction observed in rotifers across generations meets the definition of adaptive transgenerational plasticity with the exception that it is induced not by an environmental cue to which the ancestor is exposed, but rather because the ancestor has gone through diapause. It has been suggested that this inhibition could be triggered by the fertilisation of the oocytes of the sexual females, leading to the diapausing egg formation and to the transition through diapause therefore, this plastic effect seems to be endogenously controlled. It has been hypothesized that the transgenerational inhibitory effect on sexual reproduction observed in rotifers helps to prevent post-diapause progeny from entering diapause again when conditions are reliably favourable for population growth and colonisation of the water column. Originally, it has been proposed that this effect could be adaptive in scenarios where exiting from diapause is not synchronous among rotifer genotypes, thus allowing late-hatching

genotypes to still grow –and so not reproduce sexually and enter into diapause– when other conspecifics have already colonised the water column. According to theory, this transgenerational inhibitory effect on diapause induction in later generations would be adaptive in predictable environments. If environmental conditions are likely to become unfavourable at a predictable time towards the end of the growing season, then it would be advantageous for a genotype if its asexual offspring does not enter diapause until that time. Conversely, in an unpredictable environment, where ancestors cannot anticipate the end of favourable conditions for growth, it would be advantageous to enter diapause as soon as possible, and then the transgenerational inhibitory effect would be maladaptive and selected against.

The specific molecular mechanism underlying the transgenerational inhibitory effect on diapause induction in rotifers remains unknown. In general terms, mechanisms underlying transgenerational plastic responses may accommodate within the so-called inherited gene-regulation mechanisms. These mechanisms broadly include (1) cytoplasmatic cellular components and non-DNA-bound factors, as well as (2) genome-associated processes such as DNA methylation and histone modifications. Among the former, cytoplasmatic cellular components are endogenous components that are passed on from parents to offspring via cytoplasmatic yolk and their concentration would decrease over time or generations decreasing its concentration across generations. If this were

the case for rotifers, the inhibitory effect on sexual reproduction would manifest in each generation as long as the factor persists. 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. It has been suggested that such a factor may (1) block the reception of the protein involved in the induction of sexual reproduction or, alternatively, (2) block the production of any steroid hormone involved in sexual reproduction. In either case, this endogenous factor would promote asexual reproduction. It is worth mentioning that the concentration of this hypothetic factor that would be transferred to the next generation would depend on the number of offspring produced by a female during her reproductive period, which could act as a confounding effect. If a cytoplasmatic factor that inhibits the production of sexual females is transgenerationally transferred from mothers to daughters, it should also affect the different oocytes from the offspring of the same generation. This would mean that the sexual response to density would increase both across generations and within a generation as the concentration of the inhibitory factor decreases. Different outcomes could be expected depending on both the fecundity (i.e., the total number of offspring) of the stem female (i.e., the female hatching from the diapausing egg) and the distribution of the potential cytoplasmatic factor among the offspring, whether or not it is equally distributed, as the available amount of the factor could decrease over the

reproductive period of the mother and therefore with laying order. If the cytoplasmatic factor is evenly distributed among the offspring its concentration per daughter will decrease with the number of offspring produced. This means –assuming an equal initial amount of the cytoplasmatic factor in stem females– a higher inhibition of sexual reproduction and, thus, a lower number of sexual daughters (i.e., lower proportion of sexual reproduction, PSR) when the fecundity of the mother is low than when it is high. Alternatively, the cytoplasmatic factor could be transferred unequally, passing on the most concentration to the first daughter and decreasing with laying order. This case is more likely than the even distribution of the cytoplasmatic factor since there may be some uncertainty regarding the number of daughters that a female can produce during her lifetime. In both scenarios, the PSR would increase with laying order as the concentration of the inhibitory factor decreases. As above, the higher the fecundity, the higher the PSR. An alternative way of explaining the mechanism underlying this transgenerational effect is genome-associated processes such as DNA methylation or histones modifications. DNA methylation is considered a common epigenetic signalling mechanism for the inhibition of gene expression that may affect several generations. Interestingly, DNA methylation has been shown to be involved in the inhibition of diapause induction in some species of insects and there is some evidence that DNA methylation is involved in the regulation of genes related to hormones in organisms with complex

life cycles such as rotifers. It has also been suggested to play a role in the manifestation of interval timers, with some evidence indicating that DNA methylation regulates genes related to hormones in organisms with complex life cycles. DNA methylation is catalysed by a family of DNA methyltransferases (DNMTs). Of the three methyltransferases known in eukaryotes –DNMT1, DNMT2 and DNMT3– DNMT1 is typically involved in the maintenance of DNA methylation, while DNMT3 in establishing new methylation marks. DNMT2, by contrast, catalyses RNA methylation, specifically in tRNA. Notwithstanding, recent findings suggest that DNMT2 may also be responsible of DNA methylation in some organisms.

In this thesis, the rotifer species *Brachionus plicatilis* sensu stricto Müller 1786 was used as experimental organism. This is a species in which the transgenerational effect on the inhibition of sexual reproduction induction has recently been described and which is postulated as a more than suitable study organism. It is a common species in saline water bodies in the Iberian Peninsula, its populations being found in both inland endorheic ponds and coastal lagoons. These ponds are shallow and some of them are linked by groundwater, but their main input of water is rainfall. These habitats show a high degree of seasonality and unpredictability at several time scales specially because of their climate, hydrogeology and orography. The habitats studied in this thesis differ broadly in both the degree of environmental predictability and the hydroperiod length. Environmental predictability for *B. plicatilis*

populations focused on interannual variation in the length of the hydroperiod and was quantified based on the presence or absence of water as a state variable at a monthly time scale. This predictability index likely takes into account the point of view of the focal species (i.e., its perception of environmental fluctuation), according to the biological information available for this species. They are small aquatic invertebrates, eurioic (generalists) and able to reach high population sizes in small volumes of water. Therefore, they can cope with a wide variation in the flooding area of the waterbodies they inhabit. 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). Specifically, this thesis pays 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.

The rotifer species *B. plicatilis* is considered a model organism in evolutionary ecology research because of its small body size and very high intrinsic growth rates, which are mainly due to the short generation times it exhibits. The capacity for quick growth facilitates rapid local adaptive responses, which provide an advantage in time-varying environments. Furthermore, short generation times enable the gathering of data from many generations during affordable experimental times, making this trait

especially relevant in the context of this thesis. Furthermore, there are other important features related to the life cycle of *B. plicatilis* which are worthy to highlight. As a cyclical parthenogen, *B. plicatilis* has the capability of combining both asexual proliferation through parthenogenesis and sexual reproduction. The former allows to produce clonal lineages that can be used for educated comparisons, e.g., to study the response of a same genetic lineage under different environmental conditions and, thus, to differentiate between genetic and environmental effects on the phenotype of an organism. On the other hand, sexual reproduction in this species is induced by population density, which cand be controlled under experimental conditions. Finally, it should be noted that diapausing egg production is the result of sexual reproduction. Additionally, the mass production of diapausing eggs in the laboratory allows the possibility of initiating experimental clonal lineages from a single diapausing egg at any desired or convenient time.

To provide convincing evidence for the existence of nongenetic transgenerational effects, one of the requirements that studies must fulfil is to demonstrate that a plastic response of the relevant trait of interest is transmitted across generations. A classic approach for disentangling plastic responses –occurring both within and across generations– from genetic responses is to compare individuals of the same populations raised in a controlled, shared common environment (i.e., a common-garden experiment). By controlling for environmental differences across

populations in the common garden, it is possible to unravel the relative contributions of environmental and genetic variation of the phenotypic variation observed among populations. In common garden experiments, populations from different points of the distribution species range are exposed to the exact same environmental conditions to test whether trait differences in natural populations are retained under experimental conditions. When a common-garden experiment fails to confirm a genetic basis for trait differences, a common conclusion is that the differences among natural populations must result from plasticity. In this thesis, a variant of common garden experiment was used to differentiate plastic responses that occur both within and across generations from genetic responses in individuals from the same populations raised in controlled environments. Briefly, naturally evolved genotypes of *B. plicatilis* from the field populations were isolated in the form of diapausing eggs. These eggs were used to found laboratory populations and produce new cohorts of diapausing eggs under common conditions. Since the studied transgenerational effect implies the inhibition of sexual reproduction during a refractory period of insensitivity to sex inducing conditions that is observed for a number of post-diapause generations, the F0 generation of each experimental clone to be followed across generation in the transgenerational common garden was the stem female hatched from each of the diapausing eggs produced in the laboratory common environment. Using *B. plicatili*s, which can reproduce clonally, the same

genotypes could be tested in the common garden experiment across generations and therefore isolate the transgenerational effect under study. To assess the lasting of the inhibitory effect over generations, the sexual response was estimated through clonal generations by means of bioassays of self-induced sexual reproduction raised in standardized sex-inducing conditions. These bioassays entailed to estimate the proportion of sexual females generated among the offspring of one or several initial females in response to crowding into the culture medium.

The molecular basis underlying the nongenetic transgenerational effect was studied by real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR is a technique that has been increasingly used in recent years, linking phenotypic observations with molecular data in a quantitative way from microbiology to human or plant biology studies including also studies in monogonont rotifers. This technique allows the detection of low abundances of mRNA, being the most sensitive, specific and reproducible technique for mRNA quantification.

Objectives

This thesis addresses the key theoretical prediction of whether environmental predictability influences a transgenerational effect in rotifers. It takes advantage of a well-studied habitat–population system

of rotifer populations inhabiting small brackish ponds in Eastern Spain displaying a wide gradient in environmental predictability.

The main goals of the thesis are: (1) To study the nongenetic transgenerational inhibitory effect on the sexual response to population density in relation to the degree of environmental predictability experienced by natural populations of the rotifer *B. plicatilis*, (2) to address the molecular basis of this transgenerational effect by tracking, along successive generations, the expression levels of genes related to the synthesis of sex hormones and a potential epigenetic signalling mechanism in clones of *B. plicatilis* populations from habitats with different levels of environmental predictability, and (3) to explore whether the pattern of expression of the sexual reproductive inhibition trait in *B. plicatilis* across generations and among the offspring of the same generation is consistent with the transference of a hypothetical cytoplasmatic endogenous factor as the molecular mechanism underlaying the transgenerational effect.

Outline of the thesis: methodology and main results

The thesis, in the parts following the general introduction (**Chapter 1**), was organized as follows.

Chapter 2 provides the methodological context of the thesis by describing in detail the model organism used, the rotifer *B. plicatilis*, and the study

system. It also outlines the basics of the experimental approach used to study transgenerational effects and their possible underlying mechanisms in the subsequent chapters of this thesis. Firstly, the biological features of *B. plicatilis* are detailed. The emphasis is placed on its life cycle –they present a cyclical parthenogenetic cycle that combines asexual and asexual reproduction, with the latter induced by population density–and life history traits that have different expression in distinct generations of the same genetic lineage. Secondly, the study system, which consists in several natural populations of *B. plicatilis* inhabiting Mediterranean ponds from Eastern Spain and that cover a wide gradient of environmental predictability, is depicted. Thirdly, the experimental design for the study of transgenerational effects used throughout the thesis is described. A variant of a common garden experiment was used to differentiate plastic responses that occur both within and across generations from genetic responses in individuals from the same populations raised in controlled environments. Briefly, naturally evolved genotypes of *B. plicatilis* from the field populations were isolated in the form of diapausing eggs. These eggs were used to found laboratory populations and produce new cohorts of diapausing eggs under common conditions. Since the studied transgenerational effect implies the inhibition of sexual reproduction during a refractory period of insensitivity to sex inducing conditions that is observed for a number of post-diapause generations, the F0 generation of each experimental clone

to be followed across generation in the transgenerational common garden was the stem female hatched from each of the diapausing eggs produced in the laboratory common environment. Using *B. plicatili*s, which can reproduce clonally, the same genotypes could be tested in the common garden experiment across generations and therefore isolate the transgenerational effect under study. To assess the lasting of the inhibitory effect over generations, the sexual response was estimated through clonal generations by means of bioassays of self-induced sexual reproduction raised in standardized sex-inducing conditions. These bioassays entailed to estimate the proportion of sexual females generated among the offspring of one or several initial females in response to crowding into the culture medium. Finally, the procedure of the molecular technique employed for exploring the genetic basis of the transgenerational effects, reverse transcription in real-time by quantitative polymerase chain reaction (RT-qPCR), is outlined. The RT-qPCR is a technique that allows the detection of low abundances of mRNA and links phenotypic observations with molecular data in a quantitative way.

In **Chapter 3,** the relationship between the nongenetic transgenerational inhibitory effect on sexual reproduction in response to population density in *B. plicatilis* and environmental predictability were investigated. According to the theory, organisms may cope with predictable environmental fluctuations that affect several generations through these

nongenetic transgenerational inhibitory effects on sexual reproduction. By contrast, these effects may be harmful in unpredictable fluctuations if diapausing eggs are not produced. This research hypothesises that in ponds where the length of the growing season is predictable, rotifer clones would reproduce asexually for more generations, allowing a fuller exploitation of the growing season and maximising the production of diapausing eggs at the end of the season. This prediction was tested by estimating the proportion of sexual females (i.e., the proportion of sexual reproduction) produced by several clones of the rotifer *B. plicatilis* inhabiting ponds that vary in the degree of predictability of the length of their growing seasons. Specifically, in this chapter, the sexual response of a number of clones from eight ponds was studied across ten generations by means of self-induction sex bioassays. During the experiment, a total of 1,860 bioassays (8 populations × 6–8 clones/population × 3 lineages/clone × 10 generations) were conducted. Rotifer clones from those the more predictable ponds showed a lower proportion of sexual reproduction from the earliest generations, increasing in the later ones. However, clones from more unpredictable ponds displayed an early onset of sexual reproduction and thus a response to population density immediately upon diapausing egg hatching. Besides, results revealed the existence of genetic variability in the transgenerational inhibition of sex among *B. plicatilis* clones within populations.

In **Chapter 4**, the molecular basis of the nongenetic transgenerational inhibitory effect on sexual reproduction was investigated. In this chapter, the expression level of genes related to the synthesis of sex hormones and to a potential epigenetic signalling mechanism (DNA methylation), were tracked along successive generations from diapausing eggs in clones of *B. plicatilis* populations inhabiting ponds with different level of environmental predictability. These genes were quantified using RT-qPCR. 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 methyltransferase DNMT2 gene (*meth*), as a candidate epigenetic mechanism of control. These genes were tracked along four asexual generations from the hatching of diapausing eggs in rotifer clones from four ponds of the study system with different degree of environmental predictability. A total of 360 bioassays (4 populations × 6–8 clones/population × 3 lineages/clone × 4 generations) were carried out. An increasing expression of *edh* across generations in clones from those the more predictable ponds was observed. This finding provides a putative role of estradiol in the induction of sexual reproduction in *B. plicatilis*. However, no differences in the expression of *meth* gene were found neither across generations nor regarding the level of environmental predictability of the original habitats of the studied populations.

In **Chapter 5,** the patterns of sexual reproduction expected under the hypothesis of a mechanism for the transgenerational inhibitory effect of sex based on the transfer of any cytoplasmatic cellular factor from mothers to daughters were studied. The patterns expected for both the vertical transfer –over a lesser or greater number of successive generations– and horizontal transfer –between different daughters of the same generation, with lesser or greater maternal fecundity– of such a hypothetical factor were formulated and put to the test experimentally. Specifically, it was tested whether distributing the factor among the offspring within a generation leads to a decrease in its inhibitory effect throughout the laying order of the offspring of a mother in the same generation, as well as across generations. This prediction was tested by quantifying the proportion of sexual reproduction in the offspring of rotifer females from the first and tenth generation after hatching from the diapausing egg in several clones of *B. plicatilis*. A total of 159 bioassays were performed as a result of combining 8 clones x 2 generations (F1 and F11) x 2-14 daughters according to their laying order. Results showed that the proportion of sexual reproduction was higher in the last generation than in the first one, confirming again the nongenetic transgenerational inhibitory effect, but no differences were observed in the sexual response across laying order in any of the generations involved. These findings do not support the hypothesis of a mechanism for the transgenerational

inhibitory effect of sexual reproduction mediated by cytoplasmatic components being transferred from mother to offspring.

In **Chapter 6**, the main results of the thesis presented in the previous chapters are discussed in a general context. This chapter also proposes directions for future research and outlines the most important conclusions.

Conclusions

The main conclusions derived from this thesis are enumerated below:

1 A non-genetic transgenerational inhibitory effect affected the response to sex inducing cues over several clonal generations after leaving diapause in *B. plicatilis* populations.

2 The transgenerational effect differed among populations from natural habitats varying in the degree of environmental predictability regarding the interannual distribution of hydroperiods.

3 Rotifer clones from more predictable ponds tended to be unresponsive to sex induction cues for more generations after leaving diapause than clones from unpredictable ponds. Therefore, the transgenerational inhibition of sexual reproduction has evolved

in populations of habitats where the length of the growing season is more predictable on an interannual scale.

4 The longer the growing season, the more prolonged was the transgenerational inhibition effect; that is, it affected a greater number of asexual generations. This suggests that the transgenerational effect would allow a fuller exploitation of the growing season, likely maximising the production of diapausing eggs at the end of the season.

5 There was substantial within-population clonal variation in the transgenerational inhibition of sex response. This genetic variation implies that potential for future adaptation exists in the studied populations.

6 Rotifer clones from unpredictable ponds did not exhibit transgenerational inhibition of sex induction. This could allow to produce diapausing eggs as soon as possible reducing the risk of extinction if the growing season is unexpectedly short.

7 RT-qPCR allowed to track expression of genes related to synthesis of sex hormones and to a potential epigenetic signalling mechanism along successive generations from diapausing eggs in clones of *B. plicatilis*.

8 Expression level of *edh* gene, related to the synthesis of estradiol, increased across generations in clones from rotifer populations inhabiting the more predictable environments. This provides a putative role of estradiol in the observed transgenerational effect.

9 The increase of *edh* gene expression across generations in populations from predictable environments was correlated with a higher investment in sexual reproduction. Therefore, at the molecular level, both the timing of sex and the investment in it can be tracked by quantifying the expression of this gene.

10 Expression level of *meth* gene did not show differences neither across generations nor regarding environmental predictability. This does not support the conjecture about a housekeeping methylation for the silencing of genes related to sexual reproduction.

11 No increase of sexual response to sex induction in offspring was detected with either laying order or fecundity of both stem females and of females a generation far away from the hatching of the diapausing egg. This does not support an underlying mechanism for the transgenerational inhibitory effect mediated by a cytoplasmatic factor being transferred from mother to offspring.

Resumen en castellano

Introducción

Los hábitats en los que viven los organismos presentan numerosos factores abióticos y bióticos que pueden variar temporal y/o espacialmente. Estas fluctuaciones ambientales son ubicuas y diversas en la naturaleza. Para persistir en estos ambientes variables, los organismos requieren estrategias fisiológicas, morfológicas, bioquímicas, comportamentales y de rasgos de la historia vital que medien en las respuestas a las fluctuaciones. Para entender estas estrategias es preciso explorar los patrones de variabilidad ambiental, los rasgos que median en las respuestas a lo largo del ciclo vital y las consecuencias de esas respuestas en la eficacia, la adaptación, la dinámica de las poblaciones y la alteración de las trayectorias evolutivas. De ahí que, tanto desde un punto de vista fundamental como aplicado, estas cuestiones se consideren centrales en ecología evolutiva.

Esta tesis se centra en hábitats que varían en el tiempo y, por tanto, en las respuestas a la heterogeneidad temporal. El modo de respuesta que se espera que evolucione en estos ambientes depende de la escala de variación temporal relativa al organismo y de la predecibilidad de dicha variación. Para los organismos que viven en ambientes temporalmente variables pero predecibles, se espera que la plasticidad fenotípica evolucione como estrategia adaptativa. Las respuestas plásticas pueden

ser reversibles o irreversibles, dependiendo de si el cambio fenotípico revierte o no a su condición original, y pueden implicar una o más generaciones. Esta tesis estudia un caso en el que el fenotipo de un individuo está bajo el control de generaciones anteriores del mismo linaje genético. Los efectos transgeneracionales no genéticos de carácter adaptativo engloban la plasticidad transgeneracional, una forma de plasticidad fenotípica que opera a través de las generaciones y por la que el fenotipo de un individuo se ve afectado por el fenotipo o el ambiente de sus antepasados. Según los estudios teóricos, el valor adaptativo de la plasticidad transgeneracional está ligado a la existencia de heterogeneidad ambiental temporal y a la capacidad de la generación parental para predecir las condiciones ambientales que experimentarán las generaciones sucesivas. Estudios teóricos y experimentales sugieren que una elevada correlación ambiental entre generaciones aumenta la predecibilidad, lo que puede favorecer la selección de efectos transgeneracionales adaptativos.

Los organismos que viven en ambientes variables en el tiempo se enfrentan al reto de tener que ajustar las diferentes etapas y eventos de su ciclo vital a la ocurrencia de condiciones favorables. Este ajuste es aún más importante para los organismos con ciclos vitales complejos (es decir, aquellos en los que los organismos atraviesan varias etapas en las que difieren en forma y/o función), especialmente aquellos que entran en una fase de resistencia o que cambian de modo de reproducción. La

selección opera para maximizar la eficacia a lo largo del ciclo vital, integrando el éxito reproductivo total de un genotipo, ya sea de un individuo o de un linaje clonal (es decir, un conjunto de organismos derivados de un único progenitor por reproducción asexual, y que son genéticamente idénticos excepto por mutación), y en consecuencia los rasgos de la historia vital son los principales componentes fenotípicos de la eficacia biológica. Por lo tanto, en ciclos de vida complejos donde varias generaciones asexuales se intercalan entre eventos de reproducción sexual, las distintas generaciones de un linaje dado experimentan solo una fracción de un ciclo ambiental de cambio. En este contexto, sería adaptativo que los ancestros influyan en los fenotipos de su descendencia asexual en función del ambiente que se espera que generaciones específicas enfrenten. En estos casos, los efectos transgeneracionales vinculan el fenotipo, o el ambiente experimentado por los antepasados, con los rasgos de la historia vital expresados en la descendencia. Un ejemplo llamativo son los llamados temporizadores biológicos ("interval timers") transgeneracionales, un fenómeno descrito en muchos insectos y ácaros en el que la expresión de algunos rasgos de la historia vital se inhibe durante varias generaciones en la descendencia de las hembras que han experimentado la diapausa. Se cree que este efecto plástico de inhibición transgeneracional se basa en un cronómetro endógeno que funciona como una especie de temporizador biológico que mide la duración de un intervalo de tiempo.

La diapausa es un rasgo común en la historia vital de los invertebrados con ciclos vitales complejos. Es una forma de latencia controlada internamente que abarca un periodo en el que el desarrollo está suspendido y se considera una adaptación para sobrevivir condiciones adversas en ambientes variables en el tiempo. La fase de diapausa es, por tanto, un fenotipo adaptativo y plástico esencial para la supervivencia de un genotipo, y puede ocurrir en cualquier etapa del ciclo vital, como huevos, embriones, larvas, pupas o adultos. Sin embargo, cada taxón presenta la diapausa en una etapa específica. La diapausa puede ser inducida antes del inicio de condiciones adversas (por ejemplo, valores extremos de temperatura, sequía, oscuridad, presencia de competidores y depredadores, etc.) o como una respuesta directa a dichas condiciones antes de que se vuelvan críticas. En el caso de la diapausa facultativa, esto requiere que los individuos sean capaces de detectar señales ambientales (como temperatura, disponibilidad de alimento, fotoperiodo, densidad poblacional, etc.) que indiquen que el ambiente ha comenzado a deteriorarse y, por lo tanto, inicien la diapausa mediante mecanismos de control endógenos. Se han descrito varios efectos transgeneracionales en la inducción de la diapausa, principalmente en insectos, crustáceos y rotíferos.

Los rotíferos de la subclase Monogononta son pequeños invertebrados acuáticos. Presentan un ciclo vital complejo que combina la reproducción asexual y sexual. Este ciclo vital se denomina convencionalmente

partenogénesis cíclica porque la reproducción es principalmente asexual con episodios de reproducción sexual concomitante que tienen como resultado la producción de huevos diapausa. En latitudes templadas, las poblaciones de rotíferos suelen ser temporales, y la estación de crecimiento (es decir, el período durante el cual una población de rotíferos está activa en la columna de agua) comienza con la eclosión de los huevos de diapausa presentes en el sedimento. De estos huevos eclosionan hembras diploides asexuales (amícticas) que se reproducen por partenogénesis ameiótica durante varias generaciones. Tras esta fase inicial de proliferación clonal, la reproducción sexual se inicia en respuesta a un estímulo ambiental específico, siendo la densidad poblacional una señal común. En el género *Brachionus*, y en otras especies, la reproducción sexual es dependiente de la densidad poblacional, y se activa en respuesta a una concentración umbral en el medio de un infoquímico producido por los propios rotíferos. Este compuesto químico desencadena que las hembras asexuales produzcan hijas sexuales (mícticas) como una fracción de su descendencia. Aunque las hembras sexuales y asexuales son morfológicamente indistinguibles, pueden diferenciarse por el tamaño y la morfología de los huevos que portan. Las hembras sexuales producen huevos haploides mediante meiosis, los cuales, si no son fertilizados, se desarrollan en machos diminutos haploides (i.e., progenéticos). La inseminación de las hembras sexuales solo es posible solo durante unas pocas horas tras su

nacimiento, mucho antes de que alcancen la madurez. Un macho puede inseminar a múltiples hembras, pero una sola inseminación es suficiente para fertilizar todos los huevos que producirá una hembra sexual. Si las hembras sexuales son inseminadas a tiempo, sus huevos fertilizados se desarrollan en embriones femeninos enquistados y latentes, que entran en diapausa convirtiéndose en los llamados huevos de diapausa (o huevos de resistencia). Estos huevos se depositan en el sedimento, donde son capaces de resistir condiciones ambientales adversas (por ejemplo, desecación, salinidad y temperatura extremas, competidores, depredadores y parásitos), permaneciendo viables durante décadas, e incluso siglos. Después de un período de latencia obligatoria de duración variable, y cuando las condiciones adecuadas vuelven a la columna de agua, una fracción de los huevos de diapausa eclosiona dando lugar a hembras asexuales que restablecen la población activa y comienza una nueva estación de crecimiento. Como algunos de estos huevos no eclosionan en la siguiente estación favorable y permanecen en los sedimentos durante períodos más largos, forman bancos de huevos de diapausa. De esta manera, la reproducción sexual es esencial para la producción de huevos de diapausa y representan la única vía para sobrevivir a condiciones desfavorables entre estaciones de crecimiento.

Varios estudios han demostrado en los rotíferos una inhibición de la capacidad de iniciar la reproducción sexual –y por lo tanto de producir huevos de diapausa– en respuesta a la densidad de población en varias

generaciones después de salir de la diapausa**.** Esta inhibición no genética de la reproducción sexual observada en los rotíferos a través de varias generaciones se ajusta a la definición de plasticidad transgeneracional adaptativa, con la excepción de que no es inducida por una señal ambiental a la que esté expuesto el ancestro, sino que se induce porque este ha experimentado la diapausa. Se ha sugerido que este efecto podría ser desencadenado por la fertilización de los ovocitos de las hembras sexuales, lo que lleva a la formación de huevos de diapausa y a la transición a través de la diapausa; por lo tanto, este efecto plástico parece estar controlado de manera endógena. Se ha planteado la hipótesis de que el efecto inhibidor transgeneracional de la reproducción sexual observado en rotíferos contribuye a evitar que la descendencia post-diapausa vuelva a entrar en diapausa cuando las condiciones son favorables para el crecimiento de la población y la colonización de la columna de agua. Originalmente, se propuso que este efecto pudiese tener un valor adaptativo en escenarios donde la salida de la diapausa no fuese sincrónica entre los genotipos de rotíferos, permitiendo así que los genotipos que eclosionan tardíamente aún puedan crecer —y, por lo tanto, evitar la reproducción sexual y la entrada en diapausa— cuando otros conespecíficos ya han colonizado la columna de agua. Según la teoría, este efecto inhibidor transgeneracional de la inducción de la diapausa en las generaciones posteriores sería adaptativo en ambientes predecibles. Si es probable que las condiciones ambientales se conviertan

en desfavorables en un momento predecible hacia el final de la estación de crecimiento, entonces sería ventajoso para un genotipo que su descendencia no entrara en diapausa hasta ese momento. Por el contrario, en un ambiente impredecible, en el que los ancestros no pueden anticipar el final de las condiciones favorables para el crecimiento, sería ventajoso entrar en diapausa lo antes posible, y entonces el efecto inhibidor transgeneracional no sería adaptativo y sería seleccionado en contra.

Aún se desconoce el mecanismo molecular específico que subyace al efecto inhibidor transgeneracional de la inducción de la diapausa en rotíferos. En términos generales, los mecanismos subyacentes a las respuestas plásticas transgeneracionales se pueden incluir dentro de los denominados mecanismos hereditarios de regulación génica. En general estos mecanismos incluyen (1) componentes celulares citoplasmáticos y factores no ligados al ADN, así como (2) procesos asociados al genoma, como la metilación del ADN y las modificaciones de las histonas. Entre los primeros, los componentes celulares citoplasmáticos son componentes endógenos que se transmiten de las madres a la descendencia a través del vitelo citoplasmático y su concentración disminuiría con el paso del tiempo o de las generaciones. Si este fuera el caso en los rotíferos, el efecto inhibidor de la reproducción sexual se manifestaría en cada generación mientras persistiera el factor. Un factor aún por determinar podría estar presente en los huevos de diapausa, actuando como un

inhibidor de la reproducción sexual en las primeras generaciones, disminuyendo su concentración y, por lo tanto, su efecto a lo largo de las generaciones sucesivas. Se ha sugerido que dicho factor podría: (1) bloquear la recepción de la proteína involucrada en la inducción de la reproducción sexual o, alternativamente, (2) bloquear la producción de alguna hormona esteroidea involucrada en la reproducción sexual. En cualquiera de los dos casos, este factor endógeno promovería la reproducción asexual. Es importante señalar que la concentración de este factor hipotético que se transferiría a la siguiente generación dependería del número de descendientes producidos por una hembra durante su período reproductivo, lo que podría actuar como un factor de confusión. Si este factor citoplasmático inhibidor d la producción de hembras sexuales se transfiere transgeneracionalmente de madres a hijas, también afectaría a los diferentes ovocitos de la descendencia de una misma generación. Esto implicaría que la respuesta sexual a la densidad aumentaría tanto entre generaciones como dentro de una generación a medida que la concentración del factor inhibidor disminuyese. Se podrían esperar diferentes resultados en función de la fecundidad (es decir, el número total de descendientes) de la hembra madre (es decir, la hembra que eclosiona del huevo de diapausa) y de cómo se distribuye el posible factor citoplasmático entre la descendencia, ya sea de manera equitativa o no, puesto que la cantidad disponible del factor podría disminuir a lo largo del período reproductivo de la madre y, por lo tanto, con el orden

de puesta. Si el factor citoplasmático se distribuye de manera uniforme entre los descendientes, su concentración por hija disminuirá con el número de descendientes producidos. Esto significa —suponiendo una cantidad inicial igual del factor citoplasmático en las hembras madres— una mayor inhibición de la reproducción sexual y, por lo tanto, un menor número de hijas sexuales (es decir, una menor proporción de reproducción sexual, PSR) cuando la fecundidad materna es baja en comparación con cuando es alta. Alternativamente, el factor citoplasmático podría transferirse de manera desigual, transfiriéndose una mayor concentración a la primera hija y disminuyendo con el orden de puesta. Este escenario parece más probable que una distribución uniforme del factor citoplasmático, ya que podría existir cierta incertidumbre con respecto al número de hijas que una hembra puede producir durante su vida. En ambos escenarios, la PSR aumentaría con el orden de puesta, ya que la concentración del factor inhibidor disminuiría progresivamente. Además, como se ha mencionado anteriormente, cuanto mayor sea la fecundidad mayor será la PSR. Una forma alternativa de explicar el mecanismo subyacente a este efecto transgeneracional son los procesos asociados al genoma, como la metilación del ADN o las modificaciones de las histonas. La metilación del ADN se considera un mecanismo común de señalización epigenética para la inhibición de la expresión génica que puede afectar a varias generaciones. Curiosamente, se ha demostrado que la metilación del ADN está implicada en la

inhibición de la inducción de la diapausa en algunas especies de insectos, y existen algunas evidencias de que la metilación del ADN está implicada en la regulación de genes relacionados con hormonas en organismos con ciclos vitales complejos como los rotíferos. También se ha sugerido que desempeña un papel en la expresión de los temporizadores biológicos, con algunas evidencias que indican que la metilación del ADN regula genes relacionados con hormonas en organismos con ciclos de vida complejos. La metilación del ADN es catalizada por una familia de metiltransferasas de ADN (DNMTs). De las tres metiltransferasas conocidas en eucariotas –DNMT1, DNMT2 y DNMT3–, DNMT1 suele estar involucrada en el mantenimiento de la metilación del ADN, mientras que DNMT3 se encarga de establecer nuevas marcas de metilación. DNMT2, por el contrario, cataliza la metilación de ARN, específicamente en el tRNA. No obstante, algunos hallazgos recientes sugieren que DNMT2 también podría ser responsable de la metilación del ADN en algunos organismos.

En esta tesis se ha utilizado como organismo experimental la especie de rotífero *Brachionus plicatilis* sensu stricto Müller 1786. En esta especie se ha descrito recientemente un efecto transgeneracional en la inhibición de la inducción de la reproducción sexual y se propone como organismo modelo de estudio. Es una especie común en cuerpos de agua salinos de la península ibérica, encontrándose sus poblaciones tanto en lagunas endorreicas continentales como en lagunas costeras. Estas lagunas son

poco profundas y algunas de ellas están conectadas mediante corrientes de agua subterráneas, pero su principal fuente de agua es la lluvia. Estos hábitats presentan un alto grado de estacionalidad e impredecibilidad en varias escalas temporales debido especialmente a su clima, hidrogeología y orografía. Los hábitats estudiados en esta tesis muestran una amplia variabilidad tanto en el grado de predecibilidad ambiental como en la duración del hidroperiodo. La evaluación de la predecibilidad ambiental de las poblaciones de *B. plicatilis* se centró en la variación interanual de la duración del hidroperiodo, y se cuantificó en función de la presencia o ausencia de agua como una variable de estado a escala mensual. Este índice de predecibilidad probablemente refleja el punto de vista de la especie focal (es decir, su percepción de la fluctuación ambiental), de acuerdo con la información biológica disponible para esta especie, ya que *B. plicatilis* es un pequeño invertebrado acuático, eurioico (generalista) y capaz de alcanzar tamaños poblacionales elevados. Por lo tanto, esta especie puede afrontar una amplia variación en el área inundada de los cuerpos de agua que habita. Cabe señalar que el enfoque en esta tesis se centra en la predecibilidad de la variación interanual en la duración de la estación de crecimiento, más que en su duración promedio (ya sea generalmente corta o larga). Específicamente, esta tesis pone atención en la predecibilidad, dado que el valor adaptativo de la inhibición transgeneracional de la reproducción sexual depende de la capacidad

para anticipar el ambiente que experimentarán las generaciones posteriores a la eclosión de los huevos de diapausa.

La especie *B. plicatilis* se considera un organismo modelo en los estudios de ecología evolutiva debido a su pequeño tamaño corporal, sus tiempos de generación cortos y sus elevadas tasas intrínsecas de crecimiento poblacional. Este último rasgo facilita respuestas de adaptación local rápidas, lo que supone una ventaja en ambientes variables en el tiempo. Además, los tiempos de generación cortos permiten obtener datos de muchas generaciones durante tiempos de experimentación razonables, lo que hace que este rasgo sea especialmente relevante en el contexto de esta tesis. Existen otras características importantes relacionadas con el ciclo vital de *B. plicatilis* que merece la pena destacar. Como partenogenético cíclico, *B. plicatilis* combina la proliferación asexual mediante partenogénesis con la reproducción sexual. La partenogénesis permite producir linajes clonales que pueden ser utilizados en estudios comparativos, por ejemplo, para estudiar la respuesta de un mismo linaje genético en diferentes condiciones ambientales. Esto facilita la distinción entre los efectos genéticos y ambientales sobre el fenotipo de un organismo. Por otra parte, la reproducción sexual en esta especie está inducida por la densidad de la población, un factor que puede controlarse en condiciones experimentales. Por último, cabe señalar que la producción de huevos de diapausa es el resultado de la reproducción sexual. Además, la posibilidad de producir huevos de diapausa de forma

masiva en el laboratorio permite iniciar linajes clonales experimentales a partir de un solo huevo de diapausa en el momento que se desee o convenga.

Para demostrar de manera concluyente la existencia de efectos transgeneracionales no genéticos, uno de los requisitos que deben cumplir los estudios es confirmar que una respuesta plástica del rasgo de interés se transmite de una generación a otra. Un enfoque clásico para distinguir las respuestas plásticas –que se producen tanto dentro de una generación como a través de las generaciones– de las respuestas genéticas, consiste en comparar individuos de las mismas poblaciones cultivados en un ambiente común controlado y compartido (es decir, mediante un experimento de jardín común). Este método controla las diferencias ambientales entre las poblaciones del jardín común y permite evaluar la contribución relativa de la variación ambiental y genética a la variación fenotípica observada entre las poblaciones. En los experimentos de jardín común, las poblaciones de distintas localidades del área de distribución de la especie se exponen exactamente a las mismas condiciones ambientales para comprobar si las diferencias en rasgos de las poblaciones naturales persisten en condiciones experimentales. Cuando un experimento de jardín común no confirma una base genética para las diferencias de rasgos, la conclusión común es que las diferencias entre poblaciones naturales son el resultado de la plasticidad fenotípica. En esta tesis, se utilizó una variante de

experimento de jardín común para diferenciar las respuestas plásticas –que ocurren tanto dentro como entre generaciones– de las respuestas genéticas en individuos procedentes de las mismas poblaciones, cultivados en ambientes controlados, con el fin de estudiar los efectos transgeneracionales. En breve, se aislaron genotipos en forma de huevos de diapausa de las poblaciones de campo de *B. plicatilis,* y que por tanto habían evolucionado en condiciones naturales. Estos huevos se utilizaron para fundar poblaciones de laboratorio y producir nuevas cohortes de huevos de diapausa bajo condiciones controladas. Dado que el efecto transgeneracional estudiado implica la inhibición de la reproducción sexual durante un período refractario de insensibilidad a las condiciones inductoras de la reproducción sexual, que se observa durante varias generaciones posteriores a la diapausa, la generación F0 de cada clon experimental que se siguió a lo largo de generaciones en el experimento transgeneracional de jardín común fue la hembra madre eclosionada de cada uno de los huevos de diapausa producidos en el ambiente común de laboratorio. Utilizando *B. plicatilis*, que puede reproducirse clonalmente, se pudieron probar los mismos genotipos a lo largo de las generaciones en el experimento de jardín común y, de este modo, aislar el efecto transgeneracional en estudio. Para evaluar la duración del efecto inhibidor a lo largo de las generaciones clonales, la respuesta sexual se estimó mediante bioensayos de autoinducción de la reproducción sexual realizados en condiciones estandarizadas que inducen la sexualidad.

Estos bioensayos consistieron en estimar la proporción de hembras sexuales generadas en la descendencia de una o varias hembras iniciales en respuesta al hacinamiento en el medio de cultivo.

Para explorar la base molecular que subyace al efecto transgeneracional no genético observado, se utilizó la reacción en cadena de la polimerasa con transcripción inversa cuantitativa en tiempo real (RT-qPCR). La RT-qPCR, una técnica que se ha venido utilizando cada vez más en los últimos años, permite vincular de forma cuantitativa las observaciones fenotípicas con los datos moleculares, en estudios desde microbiología a biología humana o vegetal, incluyendo también estudios en rotíferos monogonontes. Esta técnica es particularmente útil debido a su alta sensibilidad, especificidad y reproducibilidad para la detección y cuantificación de bajas cantidades de ARNm.

Objetivos

Esta tesis aborda la predicción teórica sobre si la predecibilidad ambiental influye en un efecto transgeneracional en rotíferos. Se hace uso de un sistema bien estudiado de poblaciones de rotíferos que habitan en pequeñas lagunas salobres en el este de España, las cuales presentan un amplio gradiente de predecibilidad ambiental.

Los principales objetivos de la tesis son los siguientes: (1) Estudiar el efecto transgeneracional no genético de inhibición de la respuesta sexual

a la densidad de población en relación con el grado de predecibilidad ambiental experimentado por poblaciones naturales del rotífero *B. plicatilis*; (2) Investigar las bases moleculares de este efecto transgeneracional mediante el seguimiento, a través de generaciones sucesivas, de los niveles de expresión de genes relacionados con la síntesis de hormonas sexuales y un mecanismo potencial de señalización epigenética en clones de poblaciones de *B. plicatilis* provenientes de hábitats con diferentes niveles de predecibilidad ambiental; y (3) Explorar si el patrón de expresión del rasgo de inhibición de la reproducción sexual en *B. plicatilis* a lo largo de generaciones y entre la descendencia de una misma generación es consistente con la transferencia de un hipotético factor citoplasmático endógeno como mecanismo molecular subyacente al efecto transgeneracional.

Esquema de la tesis: metodología y principales resultados

La tesis, en las partes que siguen a la introducción general (**Capítulo 1**), se organiza de la siguiente manera.

En el **Capítulo 2** se proporciona el contexto metodológico de la tesis describiendo en detalle el organismo modelo utilizado, el rotífero *B. plicatilis*, y el sistema de estudio. También se esbozan los fundamentos del enfoque experimental utilizado para estudiar los efectos transgeneracionales y los posibles mecanismos subyacentes en los

capítulos posteriores de esta tesis. En primer lugar, se detallan las características biológicas de *B. plicatilis*. Se hace hincapié en su ciclo vital –presentan un ciclo partenogenético cíclico que combina la reproducción asexual y la sexual, siendo esta última inducida por la densidad de población– y en los rasgos de historia vital que tienen diferente expresión en distintas generaciones de un mismo linaje genético. En segundo lugar, se describe el sistema de estudio, que consiste en varias poblaciones naturales de *B. plicatilis* que habitan en lagunas mediterráneas del este de España y que cubren un amplio gradiente de predecibilidad ambiental. En tercer lugar, se describe el diseño experimental utilizado a lo largo de la tesis para el estudio de los efectos transgeneracionales. Se empleó una variante del experimento de jardín común que permite diferenciar las respuestas plásticas, que se producen dentro y a través de las generaciones, de las respuestas genéticas en individuos de las mismas poblaciones cultivados en ambientes controlados. Brevemente, se aislaron genotipos de *B. plicatilis* de las poblaciones de campo en forma de huevos de diapausa. Estos huevos se utilizaron para fundar poblaciones de laboratorio y producir nuevas cohortes de huevos de diapausa en condiciones controladas. Dado que el efecto transgeneracional estudiado implica la inhibición de la reproducción sexual durante un período refractario de insensibilidad a las condiciones inductoras del sexo que se observa durante varias generaciones posteriores a la diapausa, la generación F0 de cada clon experimental a

seguir a través de la generación en el jardín común transgeneracional fue la hembra nacida de cada uno de los huevos de diapausa producidos en condiciones controladas de laboratorio. Utilizando *B. plicatilis*, que puede reproducirse clonalmente, los mismos genotipos podían ser probados en el experimento del jardín común a través de las generaciones y, por lo tanto, aislar el efecto transgeneracional en estudio. Para evaluar la duración del efecto inhibidor a lo largo de las generaciones, se estimó la respuesta sexual a través de generaciones clonales mediante bioensayos de reproducción sexual autoinducida criados en condiciones estandarizadas de inducción del sexo. Estos bioensayos consistieron en estimar la proporción de hembras sexuales generadas entre la descendencia de una o varias hembras iniciales en respuesta a la densidad en el medio de cultivo. Por último, se expone el procedimiento de la técnica molecular empleada para explorar la base genética de los efectos transgeneracionales, la transcripción inversa en tiempo real por reacción en cadena de la polimerasa cuantitativa (RT-qPCR). La RT-qPCR es una técnica que permite detectar bajas abundancias de ARNm y relacionar de forma cuantitativa las observaciones fenotípicas con los datos moleculares.

En el **Capítulo 3**, se investigó la relación entre el efecto transgeneracional no genético de inhibición de la reproducción sexual en respuesta a la densidad de población en *B. plicatilis* y la predecibilidad ambiental. Según la teoría, los organismos pueden hacer frente a fluctuaciones ambientales

predecibles que afectan a varias generaciones mediante estos efectos inhibidores transgeneracionales no genéticos. Por el contrario, estos efectos pueden ser perjudiciales en condiciones de fluctuaciones impredecibles si no se producen huevos de diapausa. Esta investigación plantea la hipótesis de que en las lagunas donde la duración de la estación de crecimiento es predecible, los clones de rotíferos se reproducirían asexualmente durante más generaciones, lo que permitiría una explotación más completa de la estación de crecimiento y maximizaría la producción de huevos de diapausa al final de la estación. Esta predicción se contrastó estimando la proporción de hembras sexuales (es decir, la proporción de reproducción sexual) producida por varios clones del rotífero *B. plicatilis* que habitan en lagunas que varían en la predecibilidad de la duración de su estación de crecimiento. En concreto, en este capítulo se estudió la respuesta sexual de varios clones de ocho lagunas a lo largo de diez generaciones mediante bioensayos de autoinducción de la reproducción sexual. Durante el experimento se realizaron un total de 1.860 bioensayos (8 poblaciones × 6-8 clones/población × 3 linajes/clon × 10 generaciones). Los clones de rotíferos procedentes de las lagunas más predecibles mostraron una menor proporción de reproducción sexual desde las primeras generaciones, proporción que fue aumentando en las generaciones posteriores. Sin embargo, los clones procedentes de lagunas más impredecibles mostraron un inicio temprano de la reproducción sexual y, por tanto, una respuesta a la densidad de

población inmediatamente después de la eclosión de los huevos de diapausa. Además, los resultados revelaron la existencia de variabilidad genética en la inhibición transgeneracional del sexo entre clones de *B. plicatilis* dentro de las poblaciones.

En el **capítulo 4**, se investigaron las bases moleculares del efecto transgeneracional no genético de inhibición de la reproducción sexual. En este capítulo, se rastreó el nivel de expresión de genes relacionados con la síntesis de hormonas sexuales y con un posible mecanismo de señalización epigenético (metilación del ADN), a lo largo de las sucesivas generaciones a partir de huevos de diapausa en clones de poblaciones de *B. plicatilis* que habitan lagunas con diferente nivel de predecibilidad ambiental. Estos genes se cuantificaron mediante RT-qPCR. Los genes seleccionados fueron (1) el gen de la 17-*β*-deshidrogenasa (*edh*), implicado en la síntesis de la hormona 17-*β*-estradiol en rotíferos, y (2) el gen de la metiltransferasa DNMT2 (*meth*), como candidato a mecanismo de control epigenético. Estos genes fueron rastreados a lo largo de cuatro generaciones asexuales desde la eclosión de los huevos de diapausa en clones de rotíferos procedentes de cuatro lagunas del sistema de estudio con diferente grado de predecibilidad ambiental. Se realizó un total de 360 bioensayos (4 poblaciones × 6-8 clones/población × 3 linajes/clon × 4 generaciones). Se observó un aumento de la expresión de *edh* a lo largo de las generaciones en los clones procedentes de las lagunas más predecibles. Este hallazgo sugiere un papel putativo del estradiol en la

inducción de la reproducción sexual en *B. plicatilis*. Sin embargo, no se encontraron diferencias en la expresión del gen *meth* ni a través de las generaciones ni con respecto al nivel de predecibilidad ambiental de los hábitats de las poblaciones estudiadas.

En el **Capítulo 5**, se comprobaron los patrones de reproducción sexual esperados bajo la hipótesis de un mecanismo para el efecto transgeneracional de inhibición del sexo basado en la transferencia de un factor celular citoplasmático de madres a hijas. Se establecieron y contrastaron experimentalmente los patrones esperados tanto para la transferencia vertical –a lo largo de un menor o mayor número de generaciones sucesivas– como horizontal –entre distintas hijas de una misma generación, con una menor o mayor fecundidad materna– de dicho factor hipotético. Concretamente, se comprobó si la distribución del factor entre la descendencia de una misma generación genera una disminución de su efecto inhibidor a lo largo de la puesta de la descendencia de una madre en esa generación, así como una disminución del efecto entre generaciones. Esta predicción se comprobó estimando la proporción de reproducción sexual en la descendencia de las hembras de la primera y la décima generación tras la eclosión del huevo de diapausa en varios clones de *B. plicatilis*. Se realizó un total de 159 bioensayos como resultado de combinar 8 clones x 2 generaciones (F1 y F11) x 2-14 hijas según su orden de puesta. Los resultados mostraron una mayor proporción de reproducción sexual en la última generación que en la

primera, confirmando de nuevo el efecto transgeneracional no genético de inhibición de la reproducción sexual, pero no se observaron diferencias en la respuesta sexual en relación con el orden de puesta en ninguna de las generaciones implicadas. Estos resultados no apoyan la hipótesis de un mecanismo para el efecto transgeneracional inhibidor de la reproducción sexual mediado por componentes citoplasmáticos que se transfieren de la madre a la descendencia.

En **el Capítulo 6** se discuten los principales resultados de la tesis presentados en los capítulos anteriores en un contexto general. En este capítulo también se proponen orientaciones para futuras investigaciones y se esbozan las conclusiones más importantes.

Conclusiones

A continuación, se enumeran las principales conclusiones derivadas de esta tesis:

1 Existe un efecto inhibidor transgeneracional no genético en las poblaciones de *B. plicatilis*, que afecta la respuesta a la inducción de la reproducción sexual de varias generaciones de un linaje clonal tras abandonar la diapausa.

2 El efecto transgeneracional difiere entre poblaciones naturales procedentes de hábitats con diferente grado de predecibilidad ambiental en la distribución interanual de los hidroperiodos.

3 Los clones procedentes de las lagunas más predecibles no respondieron a la señal de inducción de la reproducción sexual durante más generaciones tras la diapausa que los clones de lagunas impredecibles. Por lo tanto, la inhibición transgeneracional de la reproducción sexual ha evolucionado en poblaciones procedentes de hábitats donde la duración de la estación de crecimiento es más predecible en una escala interanual.

4 Cuanto más larga fue la estación de crecimiento, más prolongado fue el efecto transgeneracional de inhibición; es decir, afectó a un mayor número de generaciones asexuales. Esto sugiere que este efecto permitiría un mayor aprovechamiento de la estación de crecimiento, lo que probablemente maximizaría la producción de huevos de diapausa al final de la misma.

5 Se observó variación entre los clones de una misma población en la inhibición transgeneracional de la respuesta sexual. Esta variación genética implica que en las poblaciones estudiadas existe potencial para la adaptación futura.

6 Los clones de rotíferos procedentes de lagunas impredecibles no mostraron inhibición transgeneracional de la inducción del sexo. Esto permitiría producir huevos de diapausa lo antes posible, reduciendo el riesgo de extinción si la estación de crecimiento es inesperadamente corta.

7 La técnica RT-qPCR permitió rastrear la expresión de genes relacionados con la síntesis de hormonas sexuales y con un posible mecanismo de señalización epigenética en las generaciones sucesivas a partir de huevos de diapausa en clones de *B. plicatilis*.

8 El nivel de expresión del gen *edh*, relacionado con la síntesis de estradiol, aumentó a lo largo de las generaciones en clones procedentes de poblaciones de rotíferos que habitaban en ambientes más predecibles. Esto asigna un papel putativo del estradiol en el efecto transgeneracional observado.

9 El aumento de la expresión del gen *edh* a lo largo de las generaciones en poblaciones de ambientes predecibles se correlacionó con una elevada inversión en reproducción sexual en las generaciones tardías. Por tanto, a nivel molecular, tanto el

momento en que se inicia el sexo como la inversión en él pueden rastrearse cuantificando la expresión de este gen.

10 El nivel de expresión del gen *dmnt2* no mostró diferencias entre generaciones ni según la predecibilidad ambiental. Esto no apoya la hipótesis de un silenciamiento de los genes relacionados con la reproducción sexual mediante metilación.

11 No se detectó un aumento de la respuesta a la inducción de la reproducción sexual en la descendencia ni con el orden de puesta ni con la fecundidad, tanto en las hembras que eclosionan directamente del huevo de diapausa como en las de una generación alejada de dicha eclosión. Es decir, no se encontraron evidencias de un mecanismo subyacente para el efecto inhibidor transgeneracional mediado por un factor citoplasmático transferido de la madre a la descendencia.

1 Introduction

Environmental fluctuations and adaptive variation in evolutionary ecology

Habitats in which organisms live present numerous abiotic (temperature, humidity, nutrient availability…) and biotic factors (prey and predator abundance, interspecific competition…) that can vary temporally and/or spatially (Kolasa and Rollo 1991; Leibold et al. 2004; Vasseur and McCann 2007; Pearman et al. 2008; Leibold and Chase 2018). These environmental fluctuations are ubiquitous and diverse in nature. Thereby, spatial heterogeneity can occur across large or small patches with respect to life history traits of an organism, and, analogously, temporal fluctuations can occur over short or long timescales regarding its lifespan or generation time (i.e., Levins's coarse- and fine-grained environments, Levins 1968). Moreover, spatial variation can be highly located or take place on very large geographical scales, and, similarly, within the aforementioned time scales, the temporal fluctuations can occur on a regular temporal scale, as with daily or seasonal patterns of

environmental change, or on an irregular or random scale (Meyers and Bull 2002).

To persist in variable environments, organisms must have strategies, including physiological, morphological, biochemical, behavioural and life history traits that mediate responses to fluctuations. Understanding these strategies –and the ability they provide to cope with current and future variability–requires exploration of the patterns of environmental variability, the traits that mediate responses throughout the life cycle, and the consequences of those responses affecting fitness, driving adaptation, shaping population dynamics and altering evolutionary trajectories (e.g., Hiltunen et al. 2015; Melbinger and Vergassola 2015; Vázquez et al. 2017; Liu et al. 2019; Bernhardt et al. 2020; Gremer 2023). Moreover, understanding these processes is vital for predicting ecological responses to environmental change and for developing effective conservation strategies (e.g., Parmesan 2006; Lawson et al. 2015; Bonebrake et al. 2018; Waldvogel et al. 2020; McGaughran et al. 2021). Hence, from both a fundamental and applied point of view, these are considered central questions in evolutionary ecology (e.g., Wilbur et al. 1974; Meyers and Bull 2002; Parmesan 2006; Lenormand et al. 2009; Simons 2011; Sutherland et al. 2013; Vinton et al. 2022).

Populations can respond to environmental change through phenotypic variation within single individuals or among individuals in the population,

and either at a particular point in time, within a generation, or across generations (Meyers and Bull 2002). Underlying these responses to both temporal and spatial heterogeneity there are several different mechanisms (modes of response). This thesis deals with time-varying habitats and therefore with the responses to temporal heterogeneity. The mode of response that is expected to evolve depends on the timescale of variation relative to the organism and the predictability of that variation (Cohen 1967; Seger and Brockmann 1987; Botero et al. 2015; Gremer 2023). Figure 1.1 shows a schematic diagram of the different modes of response according to these two factors. If the pattern of variation is long relative to the generation time of the organism, adaptive tracking through the evolution of traits via natural selection on heritable variation is expected. For shorter timescales there is a wide range of responses, often referred to as nongenetic. The mode of response favoured depends on the degree of environmental predictability (i.e., the level of correlation between an environmental cue and future conditions). In unpredictable environments, risk-spreading strategies (bet hedging) will evolve to buffer individuals or lineages from the risk of having the wrong phenotype as the environment changes (Cohen 1966; Simons 2011; Botero et al. 2015). Bet hedging is considered

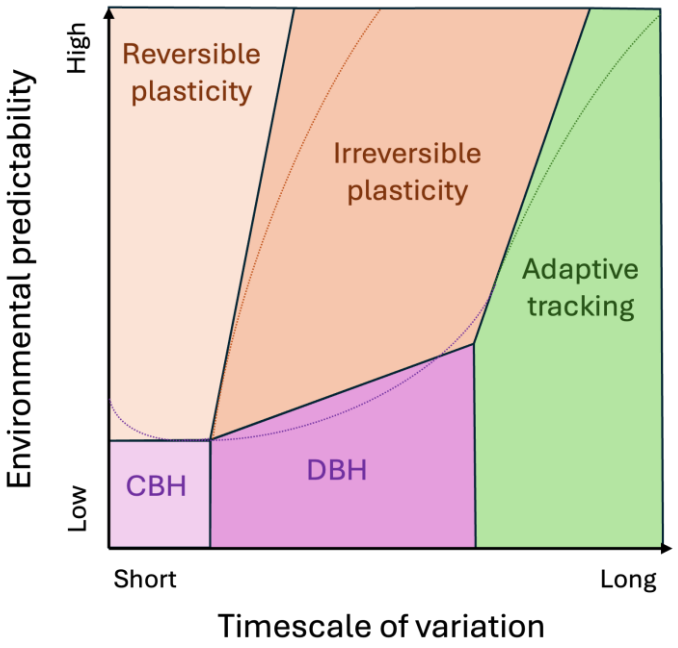


Figure 1.1. Schematic diagram of evolutionary modes of response to environmental variation under different levels of environmental predictability and relative timescale of environmental variation; modified from Botero et al. (2015). Dotted lines indicate smoothed limits for the different responses according to the original work. CBH, conservative bet hedging; DBH, diversified bet hedging. See text for details.

an evolutionary mode of response to environmental change in which variance in fitness in a genotype is reduced at expenses of a reduction in mean fitness. This can be achieved either at the individual level when a genotype expresses a low-risk phenotype whatever environmental conditions it faces (conservative bet hedging, CBH) –which is favoured at

short timescales–, or across individuals within a lineage (diversified bet hedging, DBH), if a genotype produces different phenotypes among its offspring –typically when variability occurs at longer timescales– (Slatkin 1974; Seger and Brockmann 1987; Starrfelt and Kokko 2012).

In the context of this thesis, the focus is on phenotypic plasticity, defined as the property of organisms sharing the same genotype to manifest different phenotypes in response to environmental variability (DeWitt and Scheiner 2004; Pigliucci 2005; Fusco and Minelli 2010; Reusch 2014; Sommer 2020) and it has long been considered a widespread and important mode of response for organisms living in temporally variable, but predictable environments (Botero et al. 2015; Tufto 2015; Caro et al. 2016; McNamara et al. 2016; Leung et al. 2020; Ivimey-Cook et al. 2023). The scheme in Figure 1.1 differentiates between reversible and irreversible plastic responses, depending on whether the phenotypic change reverts to its original condition. Again, the degree of reversibility seems to be associated with the scale at which environmental changes take place in relation to the generation time of the organism in focus. Thus, reversible plasticity would take place on the scale of within-generation variation (Gremer 2023), with single individuals adaptively matching their phenotype to the environment when reliable cues allow to predict the forthcoming selective environment (Reed et al. 2010; Beldade et al. 2011; Chevin and Lande 2015; Bonamour et al. 2019). Alternatively, an individual's phenotype may be under the control of prior

generations (transgenerational plastic effect), so that the ancestor's phenotype determines the phenotype produced by a given genotype of the offspring (e.g., Chevin et al. 2010; Bonduriansky et al. 2012; Leimar and McNamara 2015; Colicchio and Herman 2020; Diaz et al. 2021). For these cases involving several generations, it seems obvious that the persistence of the induced phenotypic changes must be higher, if not strictly irreversible (Donelson et al. 2018).

Transgenerational effects in time-varying environments

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 and Day 2020). The importance and commonness of transgenerational effects in shaping the phenotype and fitness of the organisms are becoming increasingly evident and have attracted growing attention in recent years (e.g., Mousseau and Fox 1998a, 1998b; Uller 2008; Badyaev and Uller 2009; Bonduriansky and Day 2009, 2018; Jablonka and Raz 2009; Danchin et al. 2011; Day and Bonduriansky 2011; Uller and Pen 2011; Bonduriansky et al. 2012; Danchin 2013; Uller et al. 2013; Bell and Hellmann 2019; Bonduriansky 2021). These studies address various aspects, including the prevalence of these effects in nature, the nongenetic mechanisms that

determine their inheritance, and the way they underlie and influence many ecological and evolutionary processes (Agrawal et al. 1999; Jablonka and Lamb 2005; Day and Bonduriansky 2011; Baugh and Day 2020; Pfennig 2021).

The potential adaptive value of transgenerational effects is one of the most interesting recent questions in ecology and evolution (Leimar and McNamara 2015; Proulx and Teotónio 2017; Baugh and Day 2020). However, nongenetic transgenerational effects do not always lead to enhance fitness (Agrawal et al. 1999; Marshall and Uller 2007; Burgess and Marshall 2014; Lane et al. 2015; Guillaume et al. 2016; Bonduriansky and Day 2018; Donelson et al. 2018), and hence the term “adaptive” is used thereafter here to mean that the induced phenotypic change increases individual fitness. Adaptive nongenetic transgenerational effects encompass transgenerational plasticity, a form of phenotypic plasticity operating across generations whereby the phenotype of an individual is affected by the phenotype or the environment of its ancestors (reviewed by Mousseau and Fox 1998a; Agrawal et al. 1999; Uller 2008; Bonduriansky and Day 2009, 2018; Mousseau et al. 2009; Herman and Sultan 2011; Salinas et al. 2013; Uller et al. 2013; Donelson et al. 2018; Bonduriansky 2021).

According to theory, the adaptive value of transgenerational plasticity is linked to the existence of time environmental heterogeneity and the

ability of the parental generation to predict the environmental conditions that the successive generations will experience (Mousseau and Dingle 1991; Agrawal et al. 1999; Uller 2008). Thus, as stated above, environmental predictable variation has been proposed as the underlying ecological context in which the emergence of adaptive transgenerational plasticity is possible (Uller 2008; Uller et al. 2013; Engqvist and Reinhold 2016; Proulx and Teotónio 2017). When cues informing environmental changes in time-fluctuating environments are reliable –allowing parents or even more remote ancestors to accurately predict the environment in which their offspring will most likely develop and live– the ancestor generation can increase fitness by strategically producing offspring in subsequent generations with the phenotype that confers greater reproductive value (Mousseau and Dingle 1991; Agrawal et al. 1999; Uller 2008; Herman and Sultan 2011; Dantzer et al. 2013; Donelan et al. 2020). Theoretical and experimental studies suggest that a high environmental correlation across generations enhances predictability, which can drive the selection for adaptive transgenerational effects (e.g., Lachmann and Jablonka 1996; Marshal and Uller 2007; Bonduriansky and Day 2009; Uller et al. 2013, 2015; Burgess and Marshall 2014; Rivoire and Leibler 2014; Kuijper and Hoyle 2015; Leimar and McNamara 2015; Proulx and Teotónio 2017; Colicchio and Herman 2020; Bernhardt et al. 2020; Dury and Wade 2020; Rescan et al. 2020; Yin et al. 2022). The sign of the environmental correlation is expected to correspond to the sign of the

transgenerational effect. This means that with a positive correlation, ancestors prepare their offspring for the same environment they are experiencing; with a negative correlation, they prepare them for an opposite environment; and with zero correlation, there is no significant influence on the offspring's phenotype (Lind et al. 2020).

Organisms living in time-varying environments are challenged to schedule the timing of the different stages and events of the life cycle with the occurrence of favourable conditions (e.g., Visser et al. 2010; Park and Post 2022). This scheduling is even more relevant for organisms with complex life cycles (i.e., those in which organisms undergo several stages that differ in form and/or function), especially those entering a resistance phase or switching between modes of reproduction. Selection acts to maximize fitness throughout the life cycle, encompassing the entire reproductive performance of a genotype –whether it is an individual or a clonal lineage (i.e., a set of organisms derived from a single parent by asexual reproduction, and which are genetically identical except by mutation)– and whereby the life history traits are the major phenotypic components of fitness (Stearns 1992, 2000; Roff 1992). In complex life cycles where several asexual generations are interspersed between sexual reproductive events, the different generations of a given lineage experience only a fraction of an environmental cycle of change. Therefore, it would be adaptive for ancestors to influence the phenotypes of their asexual offspring depending on the environment that specific

generations are expected to encounter. In such cases, transgenerational effects have the potential to significantly affect phenotypic traits of offspring across life-cycle stages (Marshall 2008), linking the phenotype or the environment experienced by the ancestor to the offspring life history traits. This linkage is expected to be highly advantageous and favoured by selection. A striking instance of this are the so-called transgenerational interval timers, a phenomenon described in many insects (e.g., aphids: Lees 1960, 1966; Dixon 1971, 1972; Margaritopoulos et al. 2002; Campbell and Tregidga 2006; Matsuda et al. 2017, 2020a, 2020b, 2023; dipterans: Zinovjeva 1978; Henrich and Denlinger 1982; hymenopterans: Boivin 1994; Reznik and Samartsev 2015) and mites (Razumova 1967; Geispitz 1968), in which the expression of some life history traits appears to be inhibited for several generations in the offspring of females that had undergone diapause. This transgenerational plastic restraining effect is thought to be based on an endogenous timekeeper, working as a kind of hourglass biological clock measuring the duration of a time interval (Rensing et al. 2001).

Nongenetic adaptive effect of transgenerational inhibition on diapause in predictable environments

Diapause is a common life history trait among invertebrates with complex life cycles (e.g., Cáceres 1997; Alekseev et al. 2007; Withers and Cooper

2008; Denlinger 2022). It is a form of internally controlled dormancy that encompasses a suspended period of development and is considered an adaptation to survive unsuitable conditions in time-varying environments (Ricci 2001; Schröder 2005; Ragland et al. 2019; Denlinger 2023). Diapause stage is, therefore, an adaptive and plastic phenotype essential for the survival of a genotype (Brendonck and De Meester 2003; Gourbière and Menu 2009; García-Roger et al. 2014, 2017; Hand et al. 2016; Saunders 2020a; Alfaro-Tapia et al. 2021; Maebe et al. 2021). Diapause can occur at any stage of the life cycle such as eggs, embryos, larvae, pupa or adults, but each taxa exhibits diapause in a specific stage (Ricci and Pagani 1997; Gordon and Headrick 2001; Fan et al. 2013; Baumgartner and Tarrant 2016; Diniz et al. 2017; Reznik and Voinovich 2021). Diapause can be induced prior to the onset of adverse conditions (e.g., extreme values of temperature, drought, darkness, presence of competitors and predators, etc.; Begon et al. 2006; Hairston and Fox 2009) or in direct response to them before becoming critical (Cassada and Russell 1975; Hu 2007). In the case of facultative diapause, this requires individuals to recognize environmental cues (i.e., temperature, food concentration, photoperiod, population density, etc.; Hairston and Fox 2009) that signal that the environment has started to deteriorate, thereby initiating diapause by means of endogenous control mechanisms (e.g., Brendonck 1996; Hand and Podrabsky 2000; Saunders 2020a, 2020b; Zhang and Stenberg 2022).

The phase of diapause induction –during which individuals are sensitive to cues anticipating unfavourable conditions–occurs at determined stages of the life cycle, either in the diapausing individuals themselves or in the preceding generations (e.g., Schröder 2005; Alekseev et al. 2007; Saunders 2014; Tougeron 2019). Various transgenerational effects on diapause induction have been reported, mainly in insect taxa (e.g., Mousseau and Fox 1998a; Denlinger 2002; Reznik et al. 2012; Ogawa and Miura 2014; Sato et al. 2014; Reznik and Samartsev 2015; Mukherjee and Vilcinskas 2019; Tougeron et al. 2020), but also in crustaceans (Zadereev 2003; Arbaciauskas 2004; Toyota et al. 2019; Bellin and Rossi 2024) and rotifers (Gilbert 2002, 2003, 2017; Gilbert and Schröder 2004; Seudre et al. 2019). In insects, these transgenerational diapause-inducing effects have been divided into two classes (Reznik and Samartsev 2015): (1) those where maternal exposure to diapause-inducing environmental cues results in the production of diapausing progeny, and (2) those where the offspring of females that had undergone diapause are incapable or less prone to diapause induction, even under strong diapause-inducing conditions. This second class will be probably targeted at avoiding the induction of an untimely maladaptive diapause in the offspring of females that had undergone diapause (Mousseau and Fox 1998a; Denlinger 2002; Ogawa and Miura 2014). As stated in the previous section, these transgenerational plasticity effects are often manifested as interval timers and may play a crucial role in regulating diapause across

generations, ensuring that progeny do not re-enter this state until the optimal time. Among the transgenerational interval timers described in insects, one of the best-documented cases at the physiological level is that of the transgenerational inhibition of diapause in aphids. In this case a refractory period of insensitivity to diapause-inducing conditions is observed for a number of post-diapause generations (Reznik and Voinovich 2021). This effect has been described as an instance of transgenerational plasticity (Reznik and Samartsev 2015; Tougeron et al. 2020) and is hypothesized to provide an adaptive safeguarding mechanism against an improper diapause response in a period of time during which environmental conditions are usually optimal for aphid growth (Henrich and Denlinger 1982; Saunders 2020a). Other studies regarding such a kind of transgenerational effect focus on facultatively sexual rotifers of the subclass Monogononta, small aquatic invertebrates with a complex life cycle that includes asexual and sexual reproduction. This life cycle is conventionally called cyclical parthenogenesis because reproduction is mainly asexual –allowing population proliferation– with episodes of concomitant sexual reproduction that leads to diapausing egg production (i.e., diapause is linked to sex; see Chapter 2). The induction of sexual reproduction depends upon a specific environmental signal (Gilbert 1974, 2020), with population density being one common cue. However, there is some evidence of a transgenerational regulation of the expression of sexual response to these inducers. Several studies have

proven an inhibition in the ability to initiate sexual reproduction –and therefore to produce diapausing eggs–in response to population density in several generations after leaving diapause (Gilbert 2002, 2003, 2017; Gilbert and Schröder 2004; Seudre et al. 2019). Given the analogies with aphids, it has been suggested that a similar endogenous time keeping mechanism could be at work in the case of this transgenerational inhibitory effect on the sexual reproduction in rotifers (Gilbert 2017).

According to Gilbert (2002), the nongenetic inhibition of sexual reproduction observed in rotifers across generations meets the definition of adaptive transgenerational plasticity with the exception that, unlike most of the described cases (e.g., Bonduriansky 2021), it is not induced by an environmental cue. It has been suggested that it could be triggered by the fertilisation of the oocytes of the sexual females, leading to the diapausing egg formation and to the transition through diapause (see Gilbert 2002, 2017). Therefore, this plastic effect seems to be endogenously controlled. There are other cases of transgenerational plasticity induced by fertilisation and the concomitant diapause in rotifers that affect morphological, physiological and life history traits (e.g., presence and length of spines, amount of lipid reserves, mode of reproduction…; Gilbert 2002, 2017; Gilbert and Schröder 2004). It has been hypothesized that the transgenerational inhibitory effect on sexual reproduction observed in rotifers helps to prevent post-diapause progeny from entering diapause again when conditions are reliably favourable for

population growth and colonisation of the water column. Originally, Gilbert (2002) proposed that this effect could be adaptive in scenarios where exiting from diapause is not synchronous among rotifer genotypes, thus allowing late-hatching genotypes to still grow –and so not reproduce sexually and enter into diapause–when other conspecifics have already colonised the water column. A study using a computer simulation model showed selective advantage for an effect preventing this premature re-entry into diapause when conspecifics are present (Serra et al. 2005). But beyond that, the transgenerational inhibitory effect on diapause may be adaptive only in predictable environments. If environmental conditions are likely to become unfavourable at a predictable time towards the end of the growing season, then it would be advantageous for a genotype if its asexual offspring did not enter diapause until that time. The strategy of delaying diapause until near the end of the growing season (i.e., the period when populations are active in the water column) or until the carrying capacity of the habitat is reached has been termed bang-bang strategy (Serra et al. 2004). But, in this case, it would be mediated by a transgenerational effect that an ancestor passes on to its successive progeny. Conversely, in an unpredictable environment, where ancestors cannot anticipate the end of favourable conditions for growth, it would be advantageous to enter diapause as soon as possible, and then the transgenerational inhibitory effect would be maladaptive and selected against.

Mechanisms underlying nongenetic transgenerational effects

Mechanisms underlying transgenerational plastic responses may accommodate within the so-called inherited gene-regulation (IGR) mechanisms (Adrian-Kalchhauser et al. 2020). Although the use of the term IGR is controversial because it does not include all possible types of non-genetic inheritance (Edelaar et al. 2021), it does at least unify molecular approaches and allows some classification (Adrian-Kalchhauser et al. 2021). Thus, IGR mechanisms broadly include (1) cytoplasmatic cellular components and non-DNA-bound factors –such as noncoding RNAs, nutrients, hormones and other metabolites (Anastasiadi et al. 2021)– as well as (2) genome-associated processes such as DNA methylation and histone modifications (Jablonka and Raz 2009).

Cytoplasmatic cellular components are endogenous components known to affect the physiological phenotype of offspring in several organisms (Schwabl 1993; Groothuis et al. 2005; Hasselquist and Nilsson 2009; Schröder and Gilbert 2009; Ho and Burggren 2010). Broadly, in this type of nongenetic inheritance, these components are passed on from parents to offspring (Moore and Johnston 2008; Edelaar et al. 2021) via cytoplasmatic yolk (e.g., during oogenesis; Henrich and Denlinger 1982) and their concentration would decrease over time or generations. As the concentration of these cytoplasmatic components decreases, so does their effect. It is uncertain whether the molecular mechanism underlying

interval timers in aphids is a transfer of such a cellular component, and some authors have discussed a possible role for the juvenile hormone, whose concentration would be diluted from stem females through generations until it falls below a critical threshold (Tagu et al. 2005; Ishikawa et al. 2012). A similar mechanism has been conjectured for the transgenerational inhibitory effect on sexual reproduction observed in rotifers (Gilbert 2002, 2003, 2017). Such a mechanism could be due to the transmission of a non–genome associated signalling factor being released in response to fertilisation or as a consequence of undergoing diapause. Then, this hypothetical 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 persists.

Another possibility is that these transgenerational effects –based on interval timers or similar mechanisms– are controlled by epigenetic modifications, such as DNA methylation or histone modification. These modifications regulate gene expression and can be passed on across one or more generations (Ho and Burggren 2010). In particular, DNA methylation is considered a common epigenetic signalling mechanism for inhibiting gene expression (Bird and Wolffe 1999; Bird 2002) that may affect several generations (Angers et al. 2010) and has been observed in many species (Adams 1996; Field et al. 2004). Interestingly, DNA methylation has been shown to be involved in the inhibition of diapause

induction in various species of insects (e.g., the wasp species *Nasonia vitripensis*; Pegoraro et al. 2016). It has also been suggested to play a role in the manifestation of interval timers in aphids (Matsuda et al. 2020b), with some evidence indicating that DNA methylation regulates 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 and Bestor 2005). Of the three methyltransferases known in eukaryotes –DNMT1, DNMT2 and DNMT3 (Colot and Rossignol 1999; Goll and Bestor 2005)– DNMT1 is typically involved in the maintenance of DNA methylation, while DNMT3 in establishing new methylation marks (Bestor 2000; Jaenisch and Bird 2003; Goll and Bestor 2005; Klose and Bird 2006). DNMT2, by contrast, catalyses RNA methylation, specifically in tRNA (Goll et al. 2006). Notwithstanding, recent findings suggest that DNMT2 may also be responsible of DNA methylation in some organisms (Deshmukh et al. 2018). Although little information is currently available on methylation patterns in rotifers, it is worthy to mention that a DNMT homologous to DNMT2 has been identified in certain species (Kim et al. 2016; Franch-Gras et al. 2018). This finding opens the possibility of exploring whether differences in the activity of this methyltransferase are associated with the transgenerational effect of inhibition on sexual reproduction reported in these rotifer species. To date, this has not yet been investigated.

Objectives and outline of this thesis

Even though theoretical studies have predicted environmental scenarios leading to adaptive transgenerational plasticity (Bonduriansky et al. 2012; Hoyle and Ezard 2012; Leimar and McNamara 2015), it is only recently that experimental studies have put predictions to the test (e.g., Shama 2015; Walsh et al. 2016; Lind et al. 2020; Rescan et al. 2020). However, the influence of environmental predictability on transgenerational plastic responses in nature still remains largely unexplored (Groot et al. 2017; Diaz et al. 2020; 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 and Metcalfe 2014; Engqvist and Reinhold 2016; Donelson et al. 2018).

This thesis addresses, in the framework of evolutionary ecology, the key theoretical prediction of whether environmental predictability influences a transgenerational effect in rotifers. It takes advantage of a well-studied habitat–population system of rotifer populations inhabiting small brackish ponds in Eastern Spain, which exhibit a wide gradient in environmental predictability.

The main goals of the thesis are:

(1) To study the nongenetic transgenerational inhibitory effect on the sexual response to population density in relation to the degree of

environmental predictability experienced by natural populations of the rotifer *Brachionus plicatilis* sensu stricto Müller 1786.

(2) To address the molecular basis of this transgenerational effect by tracking the expression levels of genes related to the synthesis of sex hormones and a potential epigenetic signalling mechanism across successive generations in clones of *B. plicatilis* populations from habitats with different levels of environmental predictability.

(3) To explore whether the transference of a hypothetical cytoplasmatic endogenous factor across generations could be the molecular mechanism underlaying the transgenerational inhibitory effect on sexual reproduction in *B. plicatilis*.

Considering these main objectives, the thesis is organized as follows in the sections following this general introduction (**Chapter 1**):

Chapter 2 provides the methodological context of the thesis by describing the model organism used, the rotifer *B. plicatilis*, and the study system, which consist of natural populations of this species inhabiting Mediterranean ponds with varying degrees of environmental predictability. It also outlines the basics of the experimental approach used to study transgenerational effects and their potential underlying mechanisms in the subsequent chapters. This chapter is intended to

facilitate understanding for readers who may be unfamiliar with certain aspects of the thesis.

Chapter 3 investigates the relationship between the nongenetic transgenerational inhibitory effect on sexual reproduction in response to population density in *B. plicatilis* and environmental predictability. According to the theory, organisms may cope with predictable environmental fluctuations affecting multiple generations through these nongenetic transgenerational inhibitory effects on sexual reproduction. In contrast, these effects may be detrimental during unpredictable fluctuations if diapausing eggs are not produced. This chapter examines the sexual response across ten generations in eight ponds exhibiting different degree of environmental predictability.

Chapter 4 quantifies the expression level of genes related to the synthesis of sex hormones and DNA methylation (specifically, the recently discovered methyltransferase DNMT2) in clones of *B. plicatilis.* Using reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR), this chapter seeks to understand the molecular basis of the nongenetic transgenerational inhibitory effect on sexual reproduction. These genes are tracked over several asexual generations from the hatching of diapausing eggs in rotifers inhabiting four ponds within the study system, which differ in their degree of environmental predictability.

Chapter 5 argues the expected patterns of sexual reproduction based on the hypothesis of a mechanism involving the transfer of cytoplasmatic cellular factors from mothers to daughters in the transgenerational inhibitory effect on sexual reproduction in rotifers. The expected effects of vertical transfer –over a greater or lesser number of successive generations– as well as horizontal transfer –between different daughters of the same generation, with greater or lesser fecundity– of a hypothetical cytoplasmatic factor are formulated and put to the test experimentally. Specifically, it was tested whether distributing the hypothetical factor among offspring within a generation leads to a decrease in its inhibitory effect across all offspring laid by a mother in the same generation, as well as between generations.

Chapter 6 discusses in a general context the main results of the thesis –presented in the previous chapters– proposes directions for future research, and outlines the most important conclusions.

2 The methodological context

Summary

This chapter provides an overview of the methodology used in this thesis. Firstly, the biological features of the focal species, the rotifer *Brachionus plicatilis* sensu stricto, a model organism widely used in evolutionary ecology studies, are described. The emphasis is placed on its life cycle and life history traits, which exhibit different expression across sucessive generations within the same genetic lineage. Secondly, the study system –*B. plicatilis* natural populations inhabiting ponds from Eastern Spain covering a wide gradient of environmental predictability– and the rationale for its choice, are depicted. Thirdly, the experimental design for the study of transgenerational effects used throughout the thesis is described in general terms. Lastly, the procedure of the molecular technique employed for exploring the genetic basis of the transgenerational effects, reverse transcription in real-time by quantitative polymerase chain reaction (RT-qPCR), is outlined.

The model organism of this study: the rotifer *Brachionus plicatilis*

The rotifer species *Brachionus plicatilis* sensu stricto Müller 1786 (hereafter *B. plicatilis*; Figure 2.1) is considered a model organism in evolutionary ecology research (e.g., Fussmann 2011; Snell 2014; Declerck and Papakostas 2017; Stelzer 2017; Serra et al. 2019; but see also below). This species belongs to phylum Rotifera –which means "wheel-bearer", from Latin rota ("wheel") and ferre ("to bear")– consisting in more than 2,000 species of microinvertebrates (body size mostly ranging from 100 to 500 µm, Hickman et al. 1997). Rotifers are characterized by two main anatomical features: a ciliated corona (rotatory apparatus) in the apical part of their head –which allows them locomotion and food gathering– and a mastax or muscular pharynx through which rotifers crush ingested food particles. This masticatory apparatus which contains a complex set of jaw-like structures called trophi (Wallace and Snell 1991; Segers 2008; Wallace and Smith 2009; Wallace et al. 2015; Serra et al. 2018) is used as a taxonomic feature to identify rotifer species within the same group (Koste 1978; Markevich 1989; Melone et al. 1998; Sørensen 2002; Fontaneto et al. 2004, 2007).

Rotifers are widely distributed, ranging from aquatic habitats (freshwater, brackish and marine) to the thin films of water covering mosses, lichens and liverworts, as well as soil interspaces in terrestrial

habitats (Wallace and Snell 1991; Wallace et al. 2006; Segers 2008; Wallace and Smith 2009; Fontaneto and De Smet 2015).

Figure 2.1. Photomicrograph of an asexual female of the rotifer *B. plicatilis* carrying an egg. *Source: Creative commons https://creativecommons.org/licenses/by-sa/4.0/*

Classically, the phylum Rotifera had been divided into three classes: Bdelloidea, Seisonidea and Monogononta, but its taxonomy and classification are still under study (Fontaneto and Plewka 2021). According to morphological traits (Clément 1993; Ahlrichs 1997; Ferraguti and Melone 1999) and molecular markers (Mark Welch and Meselson

2000; Sørensen and Giribet 2006; Fontaneto and Jondelius 2011; Wey-Fabrizius et al. 2014; Sielaf et al. 2016). Rotifera have a close relationship with the phylum of endoparasitic worms Acanthocephala, which has supported their grouping in the taxon named Syndermata (Ahlrichs 1997; Dunn et al. 2014; Ruggiero et al. 2015). A variety of hypotheses about the possible phylogenetic relationships between the four groups within this taxon has been suggested, but there is no consensus on which is the most feasible (Fontaneto and De Smet 2015; Fontaneto and Plewka 2021). The three classical groups differ mainly in their mode of reproduction and habitat. Bdelloidea reproduce by parthenogenesis (Flot et al. 2013), they are obligate asexuals and live in almost any humid habitat (Fontaneto and Ricci 2004). Seisonidea are obligate sexuals and live as epibionts on marine crustaceans (Ricci et al. 1993). Lastly, Monogononta are cyclical parthenogens, that is they are able to reproduce both asexually and sexually and are mostly found in the plankton of continental water bodies (Makarewicz and Likens 1979; Pace and Orcutt 1981; Segers 2008).

Most planktonic monogonont rotifers are filter feeders typically feeding on bacteria, microalgae, protozoa, yeasts and organic detritus (Wallace and Smith 2009; Wallace et al. 2015; Gilbert 2022). This, given the large abundances that their populations often reach, makes them one of the most relevant microplankton components for transferring energy and materials to upper trophic levels in the food webs of aquatic communities (Starkweather 1987; Walz 1997; Armengol et al. 2001; Gilbert 2022). The

monogonont rotifer *B. plicatilis*, the focal species of this thesis, belongs to a complex of cryptic species, which includes at least fifteen related species (Gómez 2005; Suatoni et al. 2006; Mills et al. 2017). The species of the *B. plicatilis* complex have high similarity in their morphology (Gómez et al. 2002; Ortells et al. 2003) and must therefore be recognized by means of molecular taxonomy tools (Fontaneto et al. 2007; Montero-Pau et al. 2011).

The typical life cycle of *B. plicatilis* is shown in Figure 2.2, taken as a model to illustrate cyclic parthenogenesis, which, as mentioned above, is the reproductive mode that characterises monogonont rotifers. This life cycle is complex since it combines periods of exclusive asexual reproduction (amixis) allowing population growth–with periods in which both asexual and sexual reproduction (mixis) co-occur, the latter leading to diapausing egg production. At temperate latitudes, rotifer populations are typically temporary, with their growing season (i.e., the period of time in which a rotifer population is active in the water column) starting from the hatching of diapausing eggs present in the sediment. The hatchlings of these eggs are diploid asexual (amictic) females that reproduce by ameiotic parthenogenesis for several generations. After this initial phase of clonal proliferation, the initiation of sexual reproduction depends upon a specific environmental stimulus (Gilbert 1974, 2020), with population density being one common cue. In the genus *Brachionus,* and in other species, sexual reproduction is density dependent, triggered in response

to a threshold concentration in the medium of an infochemical produced by the rotifers themselves (Carmona et al. 1993; Stelzer and Snell 2003, 2006; Snell et al. 2006). The infochemical triggers asexual females to produce sexual (mictic) daughters as a fraction of their offspring. Sexual and asexual females are morphologically indistinguishable, but they can be differentiated by the size and morphology of the oviposited eggs they carry (Carmona and Serra 1991; Carmona et al. 1995). Sexual females produce haploid eggs by meiosis, which, if not fertilised, develop into haploid progenetic dwarf males (100 µm body length in comparison to 300 µm of females, Ciros-Pérez et al. 2001). In addition to this marked sexual size-dimorphism, males of genus *Brachionus* are structurally simpler, for instance they lack a functional digestive system and do not feed (Ricci and Melone 1998; Wallace et al. 2006; Leasi et al. 2010; Fontaneto and De Smet 2015). Males have shorter lifespan than females, for instance, the lifespan of *Brachionus* males at 25 °C is only 1.5–2 days in comparison with 6–13.5 days for the females (King and Miracle 1980). Male-female encounter is random (Snell and Garman 1986). Female insemination is possible for only a few hours after birth, quite a bit earlier than maturity. A male can inseminate more than one female, but a single insemination is enough to fertilize all the eggs a sexual female will produce (Snell and Childress 1987). If sexual females are inseminated while they are still young, their fertilised eggs develop into encysted dormant female embryos, which enter diapause becoming the so-called

diapausing (or resting) eggs. These eggs settle in the sediments where they can resist adverse environmental conditions (e.g., desiccation, extreme salinity and temperature, competitors, predators, parasites; Ricci 2001), remaining viable for decades, even centuries (Marcus et al. 1994; Kotani et al. 2001; García-Roger et al. 2006). After an obligate period of dormancy of variable length (Hagiwara and Hino 1989; Martínez-Ruiz and García-Roger 2015; Schröeder 2005), and when suitable conditions resume in the water column, a fraction of the diapausing eggs hatches into asexual females to restore the active population and a new growing season begins. Nevertheless, some of these eggs do not hatch in the following favourable season after settlement and remain in the sediments for longer periods of time (Evans and Dennehy 2005), conforming a diapausing egg bank similar to the seed bank of plants (Marcus et al. 1994; Kotani et al. 2001; García-Roger et al. 2006). In this manner, sexual reproduction is key to produce diapausing eggs, representing the only way to survive unsuitable conditions between growing seasons (Serra et al. 2018).

The capacity to produce diapausing eggs resistant to adverse conditions over long periods of time –together with the ability to colonise and reproduce rapidly– seems to allow long-distance passive dispersal of *B. plicatilis*, in view of its widespread distribution (Gómez et al. 2002; Mills et al. 2017; Fontaneto 2019).

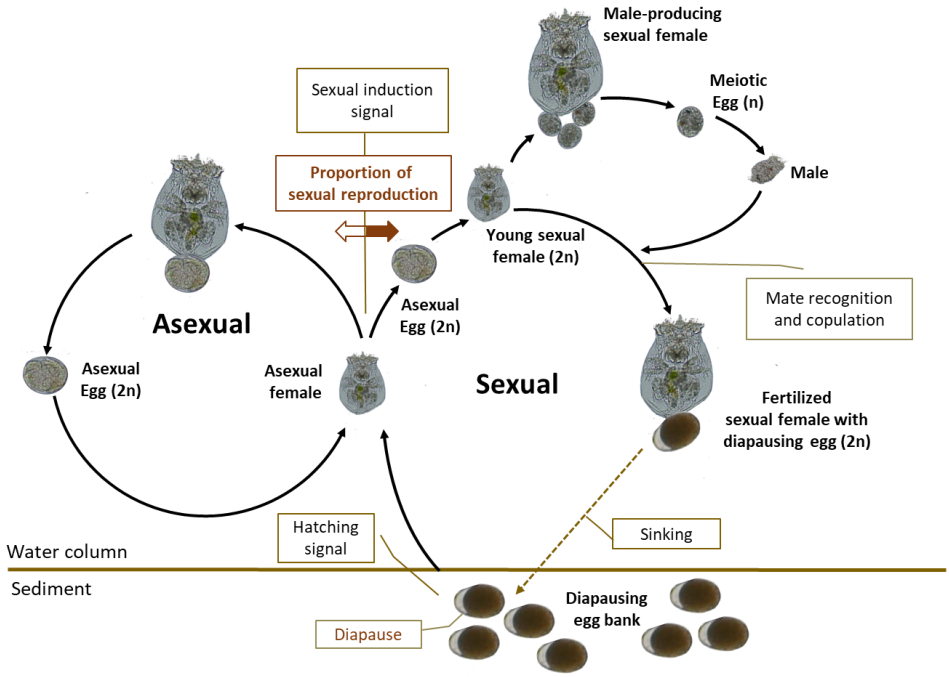


Figure 2.2. Life cycle of monogonont rotifers illustrated for the case of *B. plicatilis*. The proportion of sexual reproduction (i.e., the fraction of sexual daughters produced by asexual mothers once sexual reproduction is initiated) is the life history trait on which this thesis mainly will focus. Ploidy is indicated in brackets below each type of female or egg. *Rotifer microphotographs shown in this figure were provided by I. Jezkova*.

As most of the species within the cryptic species complex to which it belongs, *B. plicatilis* is considered cosmopolitan (Mills et al. 2017). Field studies have shown that the distribution of these species is affected

differently by a few environmental variables, mainly salinity (e.g., Gómez et al. 1995, 1997; Ortells et al. 2003; Montero-Pau et al. 2011; Papakostas et al. 2013; Walczyńska et al. 2024). Regarding *B. plicatilis*, it is an euryhaline species that can be found in environments ranging from hyposaline to hypersaline (Walker 1981; Gómez et al. 1995; Aparici et al. 2002; Ortells et al. 2003; Lowe et al. 2005, 2007), due to its osmoregulatory capacity (Lowe et al. 2005). As a result, it is regarded as the most prevalent and ubiquitous halobiont rotifer in the world (Fontaneto et al. 2006; Malekzadeh and Špoljar 2012; Zsuga et al. 2021). This species typically inhabits inland and coastal saline waterbodies, preferring relatively lower temperatures than the other species within the complex (typically below 25 °C; e.g., Gómez et al. 1995; Aparici et al. 2002; Ortells et al. 2003; Walczyńska and Serra 2022; but see also Walker 1981). Further, like most monogonont planktonic rotifers, *B. plicatilis* is a passive filter feeder, categorized as polyphagous since it eats a great variety of food items in sizes ranging from fine detritus particles (< 5 µm) to small (20–50 µm) microplankton (Gilbert 2022).

As briefly pointed out at the beginning of this chapter, the usefulness of *B. plicatilis* as a model organism in evolutionary ecology studies is being increasingly recognised (Fussman 2011; Kostopoulou et al. 2012; Declerk and Papakostas 2017; Serra et al. 2019). This species shares with the rest of monogononts several traits –some of them already described above–

in a unique combination that makes it ideal for this kind of studies. The relatively small body size (usually ~ 300 µm), allows *B. plicatilis* to be easily handled and cultured in the laboratory in small volumes. This species exhibits high intrinsic growth rates, primarily due to its short generation times, enabling it to rapidly achieve high population densities (*B. plicatilis* is able to reach densities of thousands of individuals/L short after hatching; Carmona et al. 1995, 2009). This trait facilitates rapid local adaptive responses, which provide an advantage in time-varying environments. Furthermore, short generation times allow data collection across multiple generations within feasible experimental timeframes, making this trait particularly advantageous in the context of this thesis. The broad ecological adaptability of *B. plicatilis* –as an eurioic and polyphagous species (see above)– facilitates its cultivation and maintenance in continuous and semi-continuous cultures under controlled laboratory conditions (Carmona et al. 1993; Stelzer and Snell 2003, 2006; García-Roger et al. 2009; Tarazona et al. 2017). For the purposes of this thesis, there are three additional features of its complex life cycle are particularly noteworthy: clonal proliferation, environmental induction of sex, and the production of diapausing eggs (Serra et al. 2019). The capability of both asexual proliferation through parthenogenesis and sexual reproduction enables studies in scenarios that allow the evolution of sexual reproduction. Methodologically, asexual reproduction produces clonal lineages, which facilitate controlled comparisons; for example,

these lineages allow investigating into how a single genetic lineage responds to different environmental conditions, enabling to differentiate between genetic and environmental effects on the phenotype of an organism. Moreover, by comparing various traits among genotypes, gene expression changes or epigenetic control mechanisms in relation to variable environmental conditions can be addressed (Declerck and Papakostas 2017; Serra et al. 2019; see also the following subsection). Additionally, the induction of sexual reproduction by population density (Figure 2.2) could be controlled under experimental conditions (e.g., Carmona et al. 1993; Stelzer and Snell 2003), and thus, further delve into studies on the ecology and evolution of sex. Finally, a last trait related to the complex life cycle that stands out in these rotifers is the production of diapausing eggs as the result of sexual reproduction. These eggs accumulate in the sediments of lakes and ponds in the wild forming egg banks, and its sampling allows the recovery of genotypes from the active populations and the assessment of their genetic diversity. Further, the mass production of diapausing eggs in the laboratory allows the long-term storage in diapause of a number of genotypes under conditions inhibiting hatching, and the possibility of initiating experimental clonal lineages from a single diapausing egg at any desired or convenient time (see Serra et al. 2019). It is also noteworthy that mass production of diapausing eggs from monoclonal cultures allows for sufficient egg

quantities resulting from intraclonal reproduction to be used as experimental replicates of a clone.

Transgenerational effect on sexual reproduction and environmental predictability

As stated above, life history traits linked to the mode of reproduction make *B. plicatilis* particularly attractive as a model for evolutionary ecology studies. One of these traits, the proportion of sexual daughters over total offspring –the so-called proportion of sexual reproduction (hereafter, PSR)– has been proposed as a measure of the investment in sexual reproduction in this species (e.g., Carmona et al. 2009). The response of PSR to population density has been described by a sigmoid function, which progresses from null levels of PSR at low values of population density to a linear increase in a transition region once the population density threshold that induces sexual reproduction is reached, and then asymptotically approaches a maximum value of PSR where both sexual and asexual reproduction co-occur (Montero-Pau and Serra 2011). Notwithstanding, a transgenerational effect in the sexual reproduction response to population density has been previously described in *B. plicatilis* (Seudre et al. 2019; see Chapter 1). In some species of monogonont rotifers, 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 and Schröder 2004; Seudre et al. 2019). Among monogononts, *B. plicatilis* has emerged as an ideal candidate for further investigation of this transgenerational effect. There are two features that make *B. plicatilis* particularly suitable for testing hypotheses 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, as it affects a life history trait recognized as a key component of biological fitness in rotifers –i.e., the proportion of females in the offspring that are sexual (PSR) and thus capable to produce diapausing eggs. On the other hand*, B. plicatilis* is the rotifer species in which the most significant progress has been made in studying adaptive responses to environmental fluctuation (Franch-Gras et al. 2017a; Tarazona et al. 2017) and in the characterizing its habitats in relation to the predictable or unpredictable length of the growing season (Franch-Gras et al. 2017a, 2017b; see below). Therefore, this species is particularly well-suited for testing transgenerational effects associated with environmental predictability and for further investigating the mechanisms underlying the incomplete response to sexual reproduction induction.

Study system: natural populations of *Brachionus plicatilis* in water bodies of Eastern Spain

The rotifer *B. plicatilis* is a common species in saline lakes and ponds in the Iberian Peninsula. Its populations are found in both inland, endorheic ponds and coastal lagoons (Comín and Alonso 1988; Roselló 1993; Gómez et al. 2000, 2007). Indeed, the presence of this species has been widely documented in ecological studies conducted in coastal and endorheic ponds from Eastern Spain (Ortells et al. 2000, 2003; Gómez et al. 2000, 2007; Lapesa 2004; García-Roger 2006; Campillo et al. 2009; Montero-Pau et al. 2011; Franch-Gras 2017). In these inland ponds, *B. plicatilis* commonly co-occurs in sympatry with *Brachionus manjavacas* Fontaneto et al. 2007, another of the species belonging to the *B. plicatilis* cryptic species complex (Gómez et al. 2000, 2002, 2007; Montero-Pau et al. 2011) with which it has a remarkable morphological similitude (Fontaneto et al. 2007; Gabaldon et al. 2017).

This thesis focuses on eight *B. plicatilis* natural populations inhabiting the lagoons of Pétrola and Salobralejo, and six other smaller water bodies from the Corral-Rubio-La Higuera pond complex, all located in the province of Albacete (Table 2.1; Figure 2.3 and Figures A.1.1-A.1.8 in Appendix 1). Not all populations were studied in all chapters but experiments in some chapters (see Chapters 4 and 5) were carried out using a subset of the populations after attending to logistical reasons.

The water bodies where the populations are from show variation in their flooding area (from 0.013 to 119 ha) and in water permanence conditions, being distributed in a total area of approximately 240 km^2. They are shallow (less than 1 m depth) and saline (range from 1.8 to 54 g/L of salinity). Some of them are linked by groundwater (Gómez-Alday et al. 2014) but their main input of water is rainfall. These ponds and lakes show a high degree of seasonality and unpredictability at several time scales specially because of their climate (semi-arid with an average temperature of 14 °C and annual precipitation of ca 343 mm; Franch-Gras 2017), hydrogeology and orography.

The habitats studied in this thesis differ broadly in both the degree of environmental predictability and the hydroperiod length (Table 2.1; Franch-Gras et al. 2017b). Environmental predictability for *B. plicatilis* populations focused on interannual variation in the length of the hydroperiod and was quantified by Franch-Gras et al. (2017b) 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 at a monthly time scale. Colwell's predictability index is composed of two metrics: constancy (which measures whether a water body remains in the same state through time) and contingency (which measures the repeatability of the variation pattern between states), which are summed to give a value ranging from 0 (completely unpredictable) to 1 (completely predictable). This predictability index

–when considering only two aquatic states (presence/absence of water)– likely takes into account the point of view of the focal species, *B. plicatilis* (i.e., its perception of environmental fluctuation), according to the biological information available for this species.

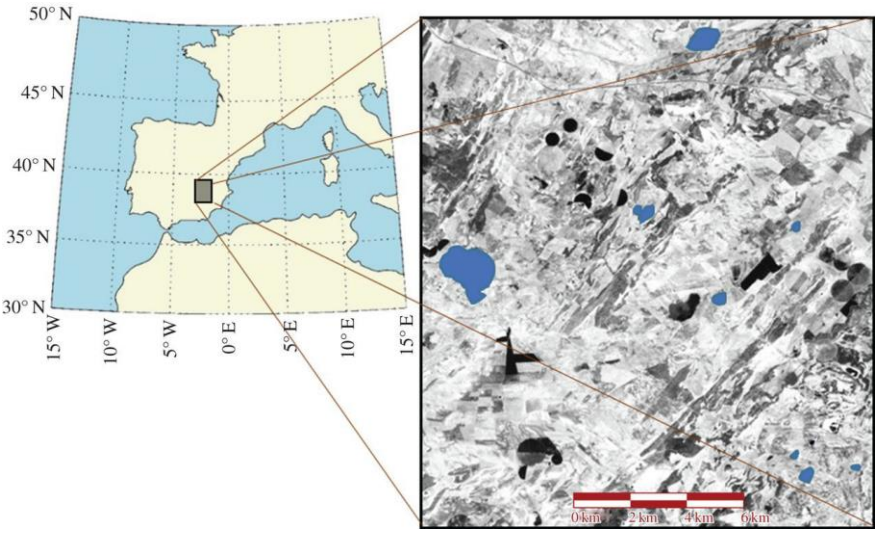


Figure 2.3. Location of the study area in Eastern Spain (38°55.4' to 38°41.803'N and 1°47.32' to 1°24.26'W). The satellite scene at right corresponds to Landsat's path 199 and row 33 and shows where the ponds (highlighted in blue) inhabited by the *B. plicatilis* populations addressed in this thesis are situated (modified from Franch-Gras et al. 2017b).

As small aquatic invertebrates that are eurioic (generalists) and capable of reaching high population sizes in small volumes of water, they can cope with a wide variation in the flooding area of the waterbodies they inhabit

(more details in Franch-Gras et al. 2017a, 2017b). 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). As it was remarked in Chapter 1, this thesis pays 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.

Experimental approach to study the transgenerational effect on sexual reproduction

To provide convincing evidence for the existence of nongenetic transgenerational effects, one of the requirements that studies must fulfil is to demonstrate that a plastic response of the relevant trait of interest is transmitted across generations (Baugh and Day 2020). While multigenerational transmission can occur across two (intergenerational) or more generations (transgenerational), multigenerational effects, when transgenerational, indicate more unequivocally that –beyond the information carried in the genetic material– some other sort of information is being faithfully transmitted over generations, as the number of generations separating the ancestral generation from the descendent generations increases (Baugh and Day 2020).

Table 2.1. Studied *B. plicatilis* natural populations, named accordingly with the ponds they inhabit, and features of their habitats (adapted from Franch-Gras et al. 2017a, 2017b).

Natural populations	**Abbreviation**	**Pond location**	**Mean water-surface area (m^2)**	**Growing-season length predictability**	**Hydroperiod (fraction of the year flooded)**
Pétrola	PET	38º50'16.82"N, 1º33'49.22"W	1,190,000	1.00	1.00
Salobralejo	SAL	38º54'52.11"N, 1º28'6.95"W	237,000	1.00	1.00
Atalaya de los Ojicos	ATA	38º46'20.97"N, 1º25'49.12"W	47,000	0.75	0.93
Hoya Turnera	HTU	38º46'31.19"N, 1º24'37.41"W	130	0.70	0.07
Hoya Yerba	HYB	38º46'46.02"N, 1º26'6.60"W	1,060	0.34	0.23
Hoya del Monte	HMT	38º50'44.87"N, 1º26'38.70"W	15,800	0.19	0.51
Hoya Chica	HYC	38º49'46.22"N, 1º27'49.74"W	32,000	0.12	0.51
La Campana	CAM	38º51'29.06"N, 1º29'36.97"W	29,000	0.11	0.63

Therefore, the experimental design used in this thesis aimed to determine the existence of a plastic response over many generations.

A classic approach for disentangling plastic responses occurring both within and across generations from genetic responses is to compare individuals of the same populations raised in a controlled, shared environment (i.e., a common-garden experiment; DeWitt and Scheiner 2004). By controlling for environmental differences across populations in the common garden, it is possible to unravel the relative contributions of environmental and genetic variation (if any) of the phenotypic variation observed among populations. In common garden experiments, populations from different points of the distribution species range are exposed to the exact same environmental conditions to test whether trait differences in natural populations are retained under experimental conditions. When a common-garden experiment fails to confirm a genetic basis for trait differences, a common conclusion is that the differences among natural populations must result from plasticity.

A variety of experimental approaches can be implemented to test for transgenerational effects with the aim to isolate progeny variation due to parental genotype from variation due to parental non-sequence-based effects (Uller et al. 2013; Donelson et al. 2018; Baker et al. 2019). Here, a transgenerational common garden design (e.g., Colicchio 2017; Toyota et al. 2019) was used to perform the experiments (Chapter 3) for assessing

the transgenerational effect on rotifer sexual reproduction (Figure 2.4). Naturally evolved genotypes of *B. plicatilis* from the field populations –inhabiting the ponds of the study system described above– were isolated in the form of diapausing eggs. These were used to found laboratory populations and produce new cohorts of diapausing eggs under common conditions. This step served to reduce confounding, undesirable effects from previous context of natural populations that were initially beyond control (e.g., age of the field isolated diapausing eggs, physiological differences of the females, etc.). Basically, the study populations were reset to a homogeneous diapause phase from which the transgenerational effect of interest previously reported in *B. plicatilis* was studied. Since this transgenerational effect implies the inhibition of sexual reproduction during a refractory period of insensitivity to the inducing conditions that is observed for a number of post-diapause generations, the F0 generation of each experimental clone to be followed across generation in the transgenerational common garden was the stem female hatched from each of the diapausing eggs produced in the laboratory common environment. Using *B. plicatili*s, a model organism capable of clonal reproduction, identical genotypes can be tested across generations in the common garden experiment. This minimizes the genetic changes that could occur from one generation to the next (as they remained limited to the occurrence of new mutations) and, therefore, the transgenerational effect under study can be isolated (see Kielland et al.

2017). To assess the lasting of the inhibitory effect over generations, the sexual response –the life history trait of interest– in each generation raised in standardized sex-inducing conditions of the common garden was estimated (see next subsection). Data on sexual response allowed to evaluate the transgenerational norm of reaction for the inhibitory effect across the studied populations (Figure 2.4).

Study of the sexual response

As stated in the previous subsection, this thesis studies the response to sexual reproduction induction across generations in *B. plicatilis.* In this species, sex induction is density dependent (Snell and Boyer 1988; Carmona et al. 1993, 1995; Stelzer and Snell 2003) and mediated by the concentration threshold of a chemical signal, a small protein (39 kDa) called mixis-inducing protein (hereafter, MIP) produced by female rotifers themselves (Snell et al. 2006). The determination of a female as asexual or sexual occurs inside the mother body before oviposition (Shull 1912; Buchner 1941; Ruttner-Kolisko 1964; Snell et al. 2006), actually during a short period in the oogenesis when the oocyte is still maturing in the germinarium (Gilbert 2003, 2007; Snell et al. 2006).

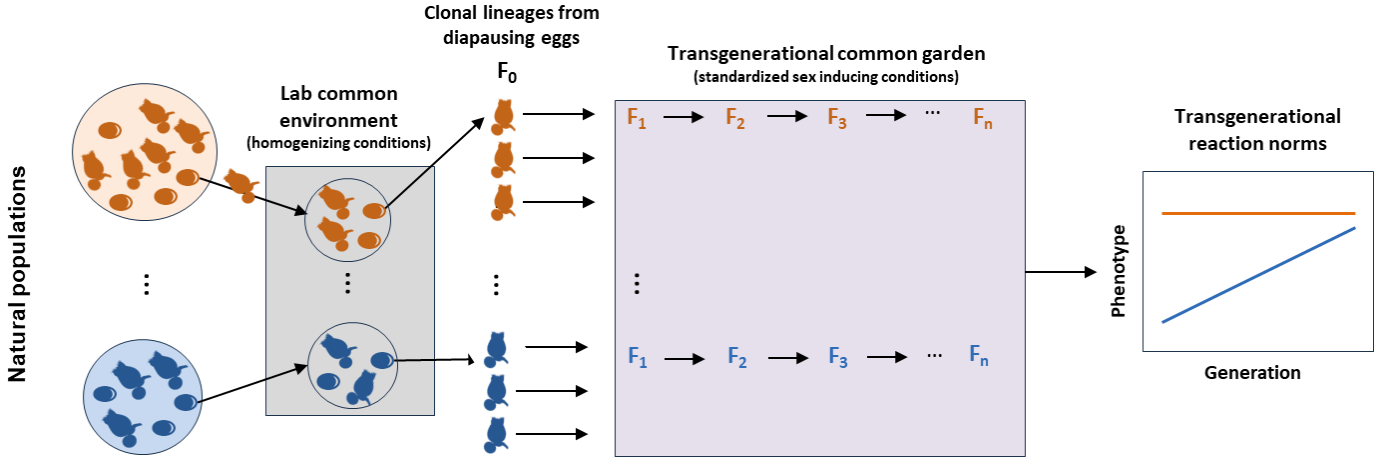


Figure 2.4. Diagram of the transgenerationally extended common garden experimental design implemented in this thesis.

The MIP may affect the oocyte directly through different pathways, (e.g., entering by diffusion to the oocyte from the vitellarium or reacting with oocyte membrane receptors; Gilbert 2003, 2007), or indirectly by affecting maternal tissues (e.g., the nervous system; Clément and Wurdak 1991), which in turn would produce transmitters acting on the oocyte to trigger sexual reproduction (Gilbert 2003). See Figure A.2 (Appendix 2) for details of the anatomy of *B. plicatilis.* Upon receiving the mixis stimulus, asexual females begin producing both sexual and asexual daughters. The production of sexual females marks the initial step in sexual reproduction and is followed by male production, fertilization, and ultimately, diapausing egg formation (see Figure 2.2). Therefore, the sexual response can be assessed by measuring any of these variables. Although some studies estimate the number of males (Snell and Hoff 1985; Snell 1986; Campillo et al. 2009) or the diapausing egg production (Lubzens et al. 1980; Lubzens 1981, 1989; Campillo et al. 2009), the proportion of sexual females (PSR) has been by far the most widely used metric (e.g., Lubzens et al. 1985; Carmona et al. 1995, 2009; Gómez et al. 1997; Ciros-Pérez et al. 2002; Hagiwara et al. 2005; Walczyńska and Serra 2014; Seudre et al. 2019) and is the response variable selected here to quantify sexual reproduction response to density in *B. plicatilis* (Chapters 3 and 5).

Several well-established bioassays in rotifers are available for studying PSR in response to the inducing chemical signal (e.g., Gilbert 1963, 2002;

Snell 1998; Snell et al. 2006). These bioassays use two different main methodological approaches. A first approach involves measuring the proportion of sexual daughters produced by females cultured in a medium in which rotifers have previously grown at a high density (conditioned medium), once the medium has been centrifuged and the supernatant microfiltered to remove rotifers, microalgae and detritus. This conditioned medium contains the sex inducer (MIP) excreted by the rotifers during population growth at a concentration well above the threshold necessary for the induction of sexual reproduction. Such bioassays, in which sexual reproduction is induced through conditioned medium have been widely employed (e.g., Gilbert 1963, 2017; Carmona et al. 1993; Yoshinaga et al. 1999; Stelzer and Snell 2003, 2006; Fussman et al. 2007; García-Roger et al. 2009; Serra et al. 2011; Seudre et al. 2019). A second methodological approach used to measure sexual response by means of bioassays are the so-called bioassays of self-induced sexual reproduction. These entail to estimate the proportion of sexual females generated among the offspring of one or several initial females in response to MIP secreted by the experimental females themselves into the culture medium (e.g., Gilbert 1963, 2002, 2004; Carmona et al. 1994; Snell and Carmona 1995; Gilbert and Diéguez 2010; Campillo et al. 2011). For instance, in some studies (Gilbert 2002, 2004), this type of bioassay has been performed by individually culturing single females and allowing them to proliferate until population densities (and therefore

concentration of MIP) reach levels sufficient to induce sexual reproduction. This thesis employs this approach, as assessing the sexual response in a population originating from a single female mimic what would occur under natural conditions, when a female hatches from a diapausing egg and proliferates by asexual reproduction in the water column. Moreover, these bioassays of self-induced sexual reproduction have been previously used in the study of the transgenerational effect in sexual reproduction in other species of rotifers (Gilbert 2002, 2004). Therefore, the use of the same methodological approach here was desirable for the sake of results comparability. On the other hand, the number of clonal genotypes studied in this thesis would have made the use of bioassays requiring the production of conditioned medium logistically unbridgeable, since it would have required maintaining a controlled high-density culture per each clone and renewing the conditioned medium daily in the bioassay cultures to prevent MIP degradation. At this sense, the bioassays of self-induced sex offered an effective and valuable alternative.

Molecular techniques

To explore the molecular basis underlying the nongenetic transgenerational effect that is the focus of this thesis, the differential gene expression in response to the stimulus inducing sexual reproduction was studied over several asexual generations within the same genotype for several clones and populations of *B. plicatilis* (further details in Chapter 4). Among the available techniques for quantifying gene expression levels, real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was selected for this study.

The RT-qPCR has gained widespread use in recent years, facilitating the quantitative linkage between phenotypic observations and molecular data in a quantitative way (Taylor et al. 2010). This technique has applications across a broad range of fields from microbiology (Di Cesare et al. 2015; Wang et al. 2016; Galafassi et al. 2022; Corno et al. 2023) to human (Thal et al. 2008; Cucuzza et al. 2017) and plant biology studies (Abdallah and Bauer 2016; Li et al. 2021), including also studies on monogonont rotifers (Denekamp et al. 2009; Snell et al. 2011, 2014). The RT-qPCR allows the detection of low mRNA abundances with the highest sensitivity, specificity and reproducibility among mRNA quantification methods (Wang and Brown 1999; Bustin 2000; Pfaffl and Hageleit 2001; Taylor et al. 2010), outperforming alternatives such as Northern blotting analysis, RNase protection assays, in situ hybridisation, or transcriptomic

sequencing analysis (Melton et al. 1984; Parker and Barnes 1999; Wang and Brown 1999; Bustin 2000; Van Straalen and Roelofs 2011; Da Fonseca et al. 2016; Tarazona et al. 2020). Absolute quantification approach in RT-qPCR enables the production of very specific and sensitive data on the expression of particular genes due to highly reproducible calibration curves (Reischl and Kochanowski 1995; Bustin 2000; Pfaffl and Hageleit 2001; Reiter et al. 2011; Pfaffl 2012). Additionally, a reference (housekeeping) gene is needed as a control to normalize the data among all conditions tested together (clones, populations, and generations in this thesis) and to quantify their expression (Huggett et al. 2005). The basis of RT-qPCR and major steps in its utilization are explained below (see Box 2.1 Glossary and Figure 2.5), with specific procedure details provided in Chapter 4.

The RT-qPCR procedure can be considered to consist of the following three phases: (1) the conversion of RNA to cDNA (reverse transcription), (2) RNA quality assessment and (3) qPCR analysis (Gibson et al. 1996; Bustin 2000). These phases are outlined in Figure 2.5. During first phase, the total RNA is extracted from biological samples (step 1.1), followed by its conversion into cDNA using oligo-dT primers that anneal to the RNA polyA tail by reverse transcription (step 1.2) (Weiss et al. 1976; Verma 1978; Hagenbüchle et al. 1979). In the second phase, RNA quality is assessed by measuring the A260/A280 ratio in aliquots from the sample. This allows to determine the presence of proteins or other contaminants

that could inhibit and alter the results, and hence gene expression in particular samples (Nolan et al. 2006, 2013; Taylor et al. 2010; Vermeulen et al. 2011; Huang et al. 2013). RNA concentration is also measured at this step, with samples showing both low RNA concentration and quality being discarded before qPCR analysis (third phase). Steps 3.1 to 3.3 include cDNA amplification by PCR, detection, and quantification, and all three are performed in the same tube. An intercalating fluorescent dye is used in the thermocycler (Higuchi et al. 1992, 1993) to detect cDNA. It emits fluorescence during DNA elongation due to binding to the new double-stranded DNA (Morrison et al. 1998; Bustin 2000; Nolan et al. 2006). The quantification of mRNA is performed in each PCR cycle through the increment of the fluorescent signal during cDNA amplification. The fluorescence signal is not observed from the start, but it becomes detectable only after a few cycles. This minimum number of cycles in which DNA concentration can be detected is referred to as the threshold cycle (Ct). Lower Ct means a higher concentration of mRNA (Nolan et al. 2006). The use of a fluorescence probe enables quantifying DNA amplification in real time (Bustin et al. 2009) without additional steps, such as agarose gel electrophoresis, to detect the products of DNA based on their size.

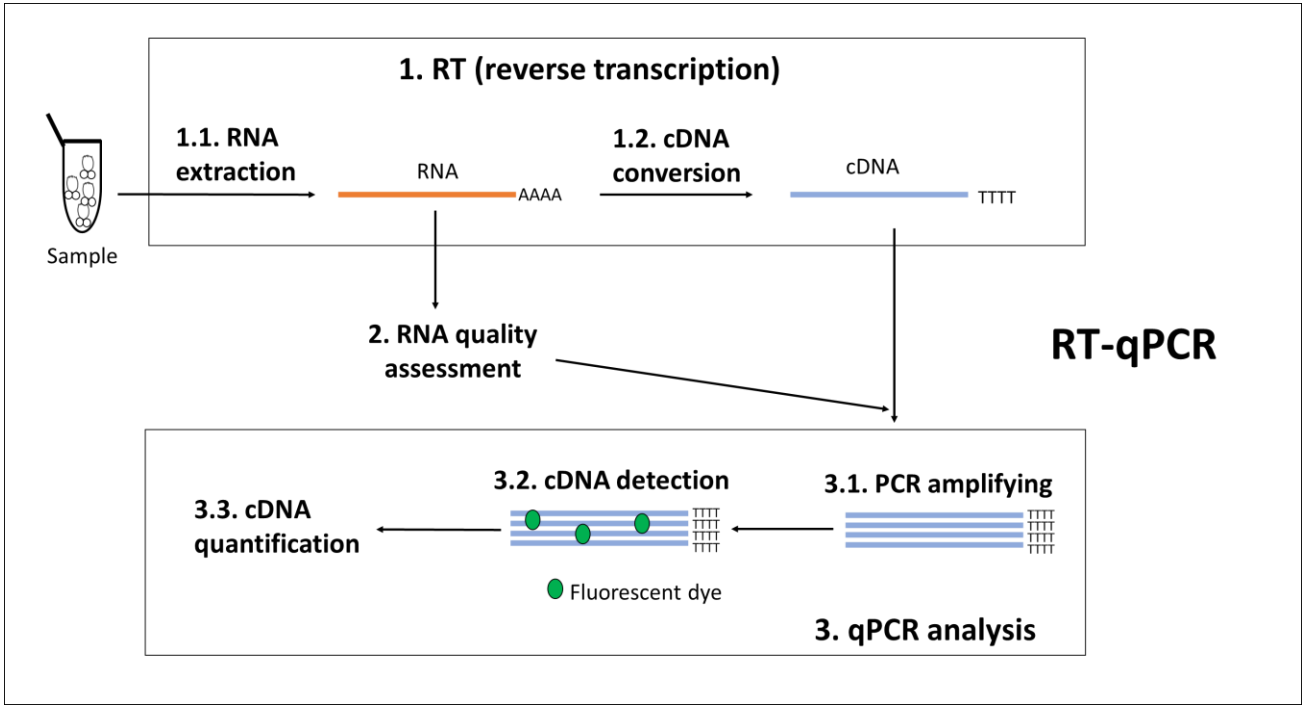


Figure 2.5. Scheme of RT-qPCR protocol (for more details see Chapter 4).

Box 2.1. Glossary

Absolute quantification: a type of RNA quantification based on using a standard or calibration curve to determine gene copy number.

Complementary DNA (cDNA): result of the conversion of mRNA via reverse transcription. It is composed of exons or coding sequences and measures gene expression.

Fluorescent dye: an intercalant dye of DNA able to detect and quantify the product of qPCR.

Messenger RNA (mRNA): result of DNA transcription that will be translated into protein using itself as a template.

Reference gene: a gene with high expression levels and no changes between samples. It is used as a control in qPCR analysis.

RNA quality: measurement of the presence of contaminants or proteins in the RNA sample, which determines its purity and integrity. It is calculated by the A260/A280 ratio. Good quality is considered when A260/A280 is 1.8–2.0.

RT-qPCR: reverse transcription in real-time by quantitative polymerase chain reaction. It is a technique which converts RNA to cDNA and quantifies it in real time.

Calibration curve: a row of dilutions from a reference sample of known concentration.

Threshold cycle (Ct): number of cycles necessary to emit the fluorescent signal and quantify cDNA concentration. High Ct means low concentration.

3 Transgenerational effects on sexual reproduction in relation to environmental predictability

Summary

An effective strategy for coping with predictable environmental fluctuations that affect several generations of individuals is through nongenetic transgenerational effects. Through these 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. This research hypothesises 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. This prediction was tested 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. 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. 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. 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). This study 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.

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. Notwithstanding, natural selection will favour the evolution of responses that allow individuals to proactively minimize such reduction in fitness. As stated in Chapter 1, 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 and 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 genotypes under a regime of fluctuating selection. However, 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 (Chevin et al. 2010; Bonduriansky et al. 2012, 2016). For these transgenerational effects to evolve, environmental changes should be reliably anticipated or vary in a predictable fashion (e.g., Herman and Sultan 2011; Leimar and McNamara 2015; Yin et al. 2019; Colicchio and Hermann 2020; Lind et al. 2020). Interestingly, nongenetic transgenerational effects may be adaptive, maladaptive or

neutral about an individual's fitness (Agrawal 1999; Burgess and Marshall 2014; Lane et al. 2015; Guillaume et al. 2016; Donelson et al. 2018).

Amongst 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 (Mousseau and Fox 1998a; Bell and Hellmann 2019). 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 (Uller 2008; Uller et al. 2013; Engqvist and Reinhold 2016; Proulx and Teotónio 2017). Due to such 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 (Mousseau and Dingle 1991; Agrawal et al. 1999; Uller 2008; Herman and Sultan 2011; Dantzer et al. 2013; Donelan et al. 2020). Under 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 in the life cycle are expected to experience (i.e., the different generations of a given lineage experience only a fraction of an environmental cycle of change). This may be the case for the transgenerational regulation of the expression of the sexual response in

some rotifer species, in which the ability to initiate sexual reproduction is inhibited several generations immediately after the hatching of the diapausing egg, even under strong sex-inducing stimulus conditions (Gilbert 2002, 2003, 2017; Gilbert and 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 Chapter 1). 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 (Serra et al. 2005; Gilbert 2017, 2020). In this chapter, it was addressed 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 explained below this study takes 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.

The eurihaline species *Brachionus plicatilis* has emerged, among monogonont rotifers, as an ideal candidate for further investigation of the adaptive value of transgenerational effects (Chapter 2). 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 recognized 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 et al. 2017a; Tarazona et al. 2017) and in the characterization of its habitats with respect to the predictable or unpredictable length of the growing season (Franch-Gras et al. 2017a, 2017b).

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 over several clonal generations to fully exploit the growing season and maximize 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 any transgenerational effect to produce diapausing eggs as soon as possible to successfully survive an unexpectedly short growing season. Accordingly, in this study, the transgenerational effects with respect to the proportion of sexual reproduction in *B. plicatilis*

populations inhabiting an unpredictable-predictable gradient were compared. As introduced in Chapter 2, data on the degree of environmental predictability of the natural habitats of a considerable number of *B. plicatilis* populations in Spain were used (Franch-Gras et al. 2017a, 2017b; Figure A.1.1-A.1.8 in Appendix 1). Thus, the specific hypothesis that the number of clonal generations during which the onset of sex is inhibited will decrease with environmental unpredictability was tested.

Material and methods

Experimental animals: Origin, identification and maintenance of parental clones

In this thesis the 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 the eight ponds and lakes of the study system described in Chapter 2. The P-clones were obtained

by sampling the sediment egg bank of the ponds. Samples from the uppermost 10 cm of sediment in each pond were obtained with a Van Veen grab (Eijkelkamp Agrisearch Equipment, Giesbeck, The Netherlands). The diapausing eggs were isolated using a sugar flotation technique (Gómez and Carvalho 2000), and those that looked healthy (García-Roger et al. 2005) were incubated individually in 96-multiwell plates containing 6 g/L saline water made with commercial sea salts (Instant Ocean®, Aquarium Systems). Plates were incubated inside a walk-in chamber with controlled conditions for hatching. These conditions were 25 °C temperature, and constant illumination with a photosynthetically active radiation (PAR) of 150–170 µmol quanta/m^2s (hereafter referred to as standard hatching conditions; see García-Roger et al. 2005, 2006; Martínez-Ruiz and García-Roger 2015). Once hatched, females were allowed to proliferate clonally in 13-mL stock cultures at controlled temperature (25 °C) and constant illumination (PAR: 35 µmol photons/m^2s). Rotifer culture medium consisted of 12 g/L saline water enriched with f/2 medium (Guillard and Ryther 1962) and containing the flagellate microalga *Tetraselmis suecica* (Kylin) Butcher 1959 (Microalgae Culture Collection of Instituto de Acuicultura Torre de la Sal-Consejo Superior de Investigaciones Científicas, IATS-CSIC, Spain) as food (800,000 cells/mL; circa. 105 mg C/L). Since these P-clones were derived from diapausing eggs collected from the field, they are representative samples of the total genotype pool of each population. Moreover, as diapausing

eggs are the product of sexual reproduction, each established clonal line represents a different genotype. In this thesis, 10 P-clones per natural population were maintained individually. Except where specified differently, pre-experimental and experimental rotifer culture media and conditions were the same as those of the stock cultures (hereafter referred to as standard culture 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.

As indicated in Chapter 2, *B. plicatilis* belongs to a cryptic species complex (Mills et al. 2017) and co-occurs in sympatry with its sibling species *Brachionus manjacavas* in many of the natural populations studied (e.g., Gómez et al. 2000, 2002, 2007; Ortells et al. 2003; Montero-Pau et al. 2011). Therefore, the parental clonal lines were identified at the species level to discriminate between *B. manjavacas* and *B. plicatilis* by genetic analysis of cytochrome c oxidase subunit I (COI) based on PCR-RFLP via two restriction enzymes, *Pvu*II and *Kpn*I (Campillo et al. 2005). The species were identified through electrophoresis in agarose since *Pvu*II produced two bands at 700 and 200 pb in *B. manjavacas,* and *Kpn*I produced a single band at 500 pb in *B. plicatilis* (Figure 3.1).

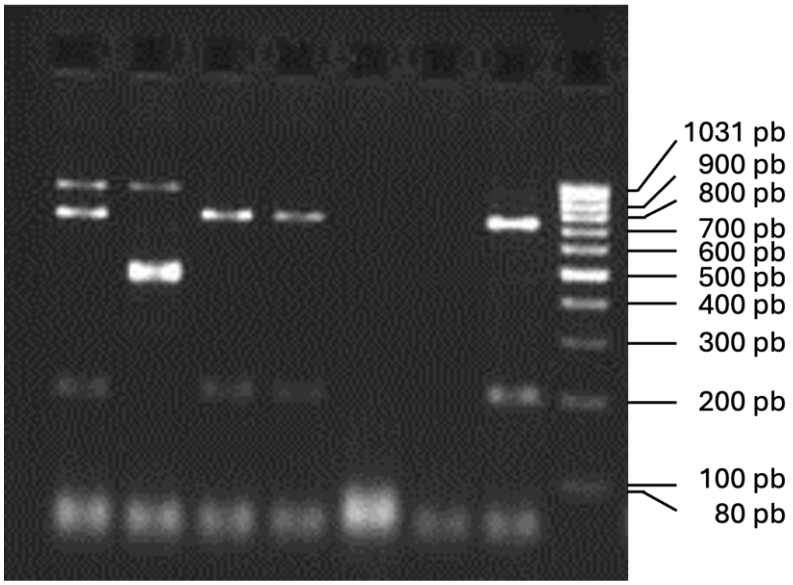


Figure 3.1. Restriction analysis of the COI gene for five rotifer clones. Bands at 700 and 200 pb are indicative of *B. manjavacas* clones (lanes 1, 3, 4 and 7). Band of 500 pb indicates a *B. plicatilis* clone (lane 2). In the first and second lines, band near 900 pb is undigested DNA. The 100-pb DNA ladder is in the last lane.

Experimental design: Transgenerational effect on sexual reproduction

Pre-experimental diapausing egg production by laboratory populations

Diapausing eggs were produced under controlled conditions in multiclonal laboratory populations generated by a mixture of P-clones to be later used in the experiments of this thesis (see Figure 3.2). From the

hatching of these new cohorts of diapausing eggs produced in the laboratory, experimental clones were obtained (see details in the next subsection), which were referred to as filial clones (hereafter F-clones). The following is a description of the diapausing egg production procedure.

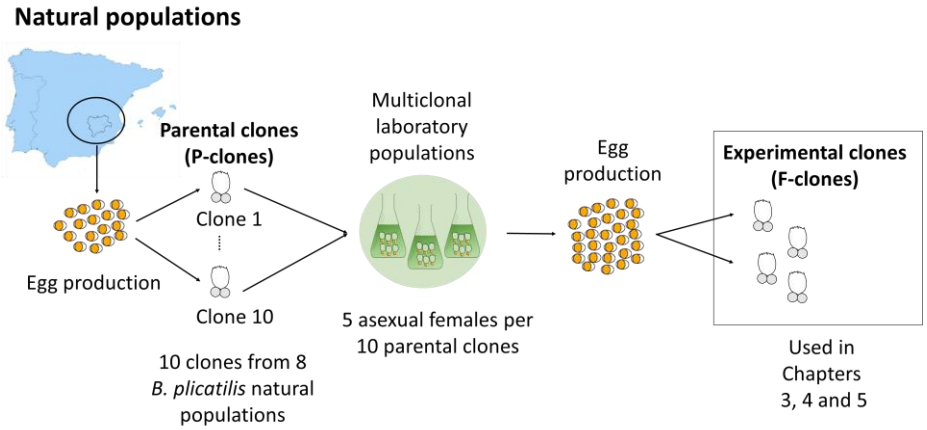


Figure 3.2. Schematic diagram of the production of experimental *B. plicatilis* clones from each natural population.

For each of the eight natural populations studied, three multiclonal laboratory populations –functioning as replicates– were generated by placing together five asexual females from each of the 10 P-clones 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 (Figure 3.2). This procedure ensured that all the harvested eggs were very close in age. The diapausing eggs were stored in saline water at 60 g/L in the dark at 4 °C (i.e., conditions known to prevent hatching; García-Roger and Ortells 2018) for at least one month to ensure the completion of the obligate period of dormancy (Hagiwara and Hino 1989; Martínez-Ruiz and García-Roger 2015) before experimental use.

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. Diapausing eggs were induced to hatch under standard hatching conditions as indicated above (García-Roger et al. 2005, 2006; Martínez-Ruiz and García-Roger 2015) and checked every 24 hr for hatchlings until an enough number (six to eleven) of stem females per population was 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) to prevent sexual reproduction induction and with sufficient food so as not to impair the fecundity of the females. For accurate control of food quality during the experiment, the microalgae used to culture rotifers were raised in a continuous flow culture in a chemostat with a dilution rate of 0.295 per day (for details, see Figure A.3 in Appendix 3).

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 3.3; Gilbert 2002; Seudre et al. 2019). 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 linage 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 (see next subsection). Due to logistical reasons, this procedure just described 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.

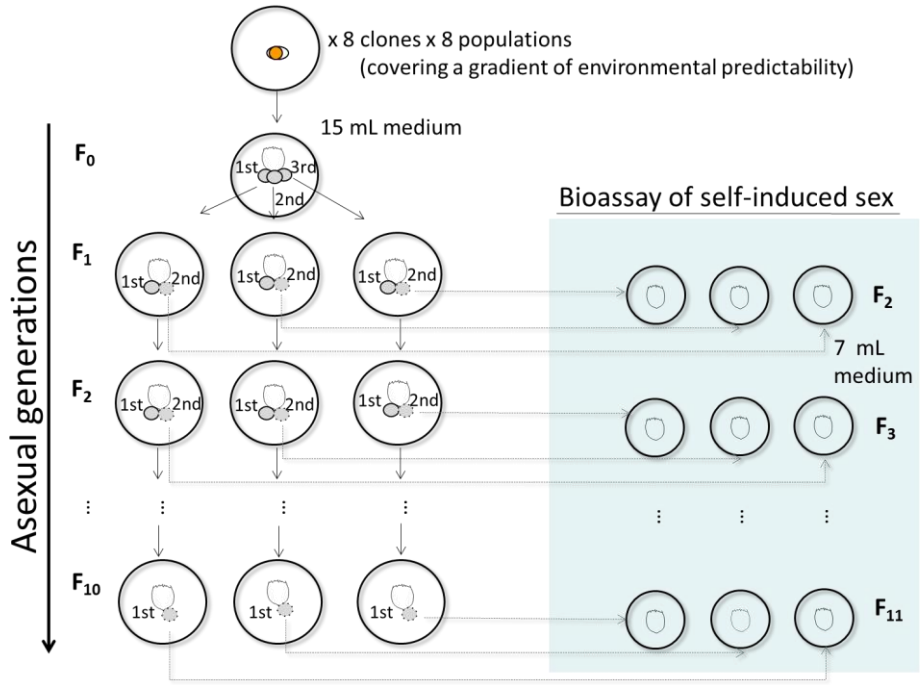


Figure 3.3. Diagram of the experimental procedure (propagation of generations and bioassay of self-induced sex) performed for each experimental F-clone.

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, 2004) 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 3.3). 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). 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%). Fixed cultures were explored under a stereomicroscope and females counted differentiating (1) gravid sexual and (2) gravid asexual females by the size and morphology of the oviposited eggs they carry (Figure 3.4A-B; Carmona et al. 1995) and Figure 3.4C, respectively), and (3) non ovigerous females (juvenile plus post reproductive; Figure 3.4D). 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.

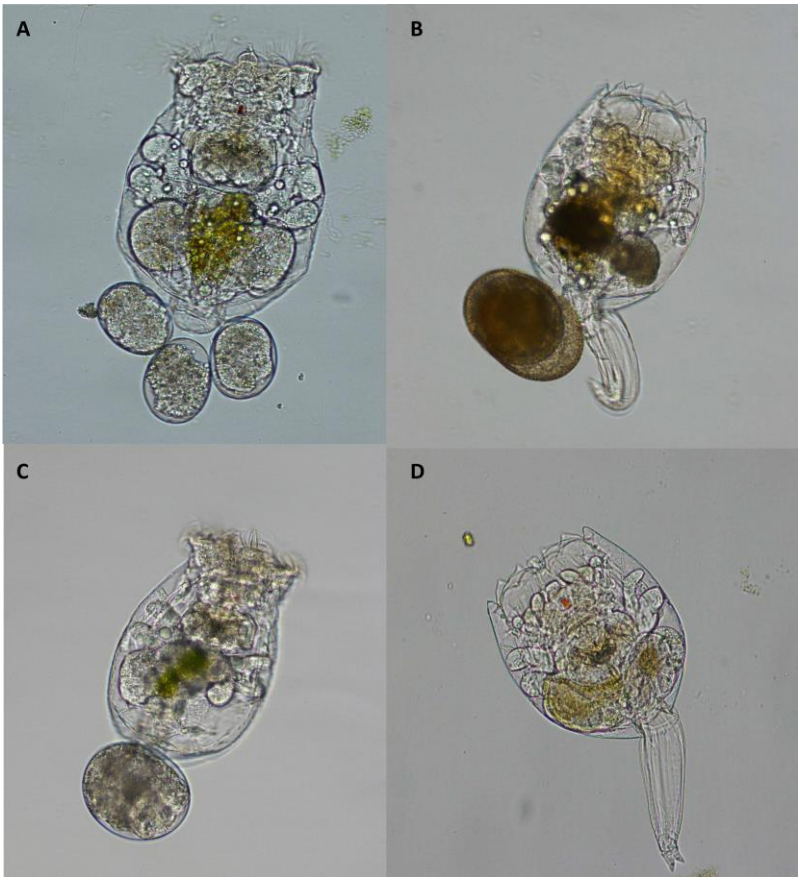


Figure 3.4. Microphotographs of different female types of *B. plicatilis*: **(A)** ovigerous, male-producing sexual female; **(B)** ovigerous diapausing egg-producing sexual female); **(C)** ovigerous, female producing asexual female; and **(D)** juvenile non-ovigerous female. Note that the images are not all on the same scale. *Source: Pictures provided by I. Jezkova*.

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, any possible effect of laying order on PSR for each generation after diapausing egg hatching was discarded through correlation analysis (Figure A.4 in Appendix 4).

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 effect. 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 Figure 2.2). 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.

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. Results showed a significant increase in PSR with generation order (Table 3.1), 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 3.1 and Figure 3.5).

A significant Generation × Predictability effect on PSR was also detected (Table 3.1). 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 values generally increasing gradually across generations (Figure 3.5).

Table 3.1. Generalised linear mixed model analysis of the proportion of sexual reproduction (PSR) in the rotifer *B. plicatilis*.

Effect	*Df*	*F*	*P*
Generation	1	50.97	< 0.001
Predictability	1	9.74	0.002
Hydroperiod	1	6.69	0.010
Predictability x Generation	1	56.49	< 0.001
Hydroperiod x Generation	1	52.24	< 0.001
Hydroperiod x Predictability	1	7.07	0.008
Generation x Predictability x 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.

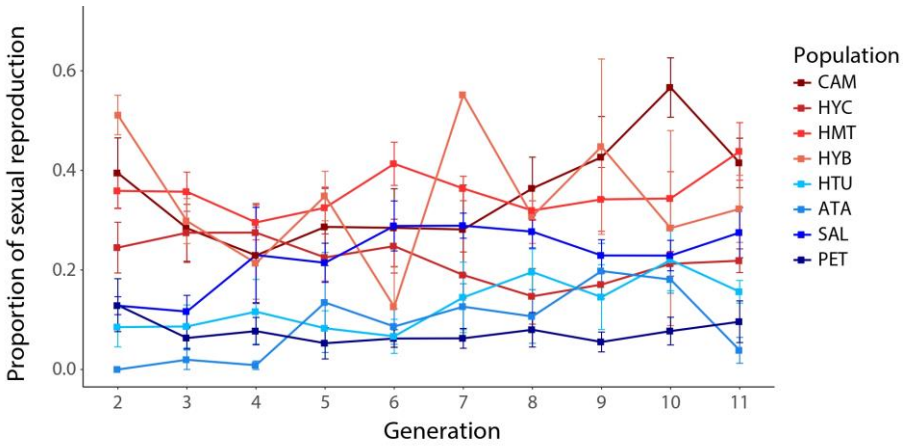


Figure 3.5. Proportion of sexual reproduction (PSR) in populations of the rotifer *B. plicatilis* across generations. The degree of predictability is coded with a gradient from darker red (rotifer populations inhabiting more unpredictable ponds) to darker blue (rotifer populations inhabiting more predictable habitats).

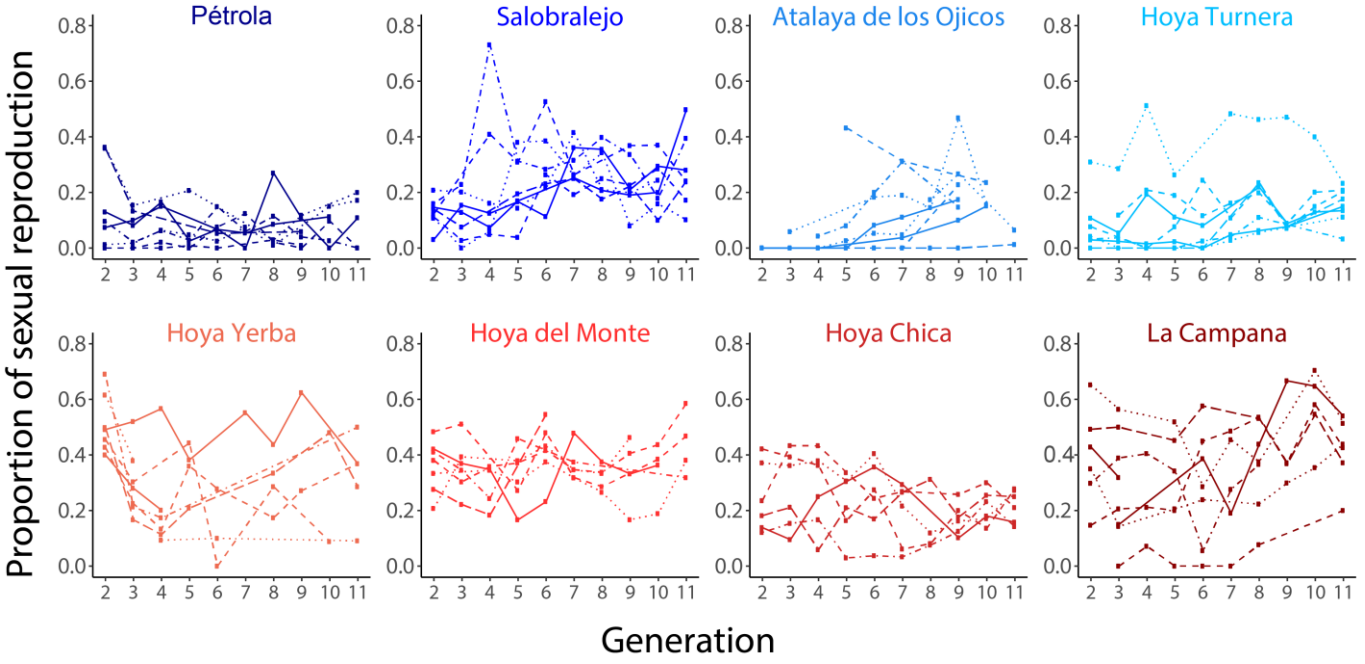


Figure 3.6. Variation in the proportion of sexual (PSR) reproduction across generations in the different clones from each population of *B. plicatilis* studied.

These results also revealed the existence of genetic variability in PSR among F-clones within populations (i.e., significant effect of the clone factor; Table 3.1), 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 3.6).

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 3.7). Figure 3.7 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 3.8). 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, results show higher PSR values than in the early generations (Table A.4 in Appendix 4).

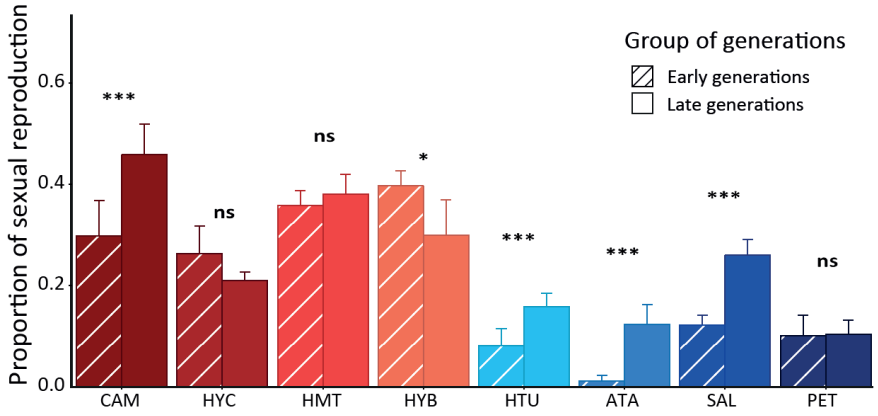


Figure 3.7. Proportion of sexual reproduction (PSR) in two groups of generations (*early*: F2 and F3; *late*: F10 and F11) in populations of the rotifer *B. plicatilis*. Populations are ordered according to the predictability of their environments. Significance was tested by means of generalised linear mixed models with binomial responses and applying Bonferroni's correction (ns: $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

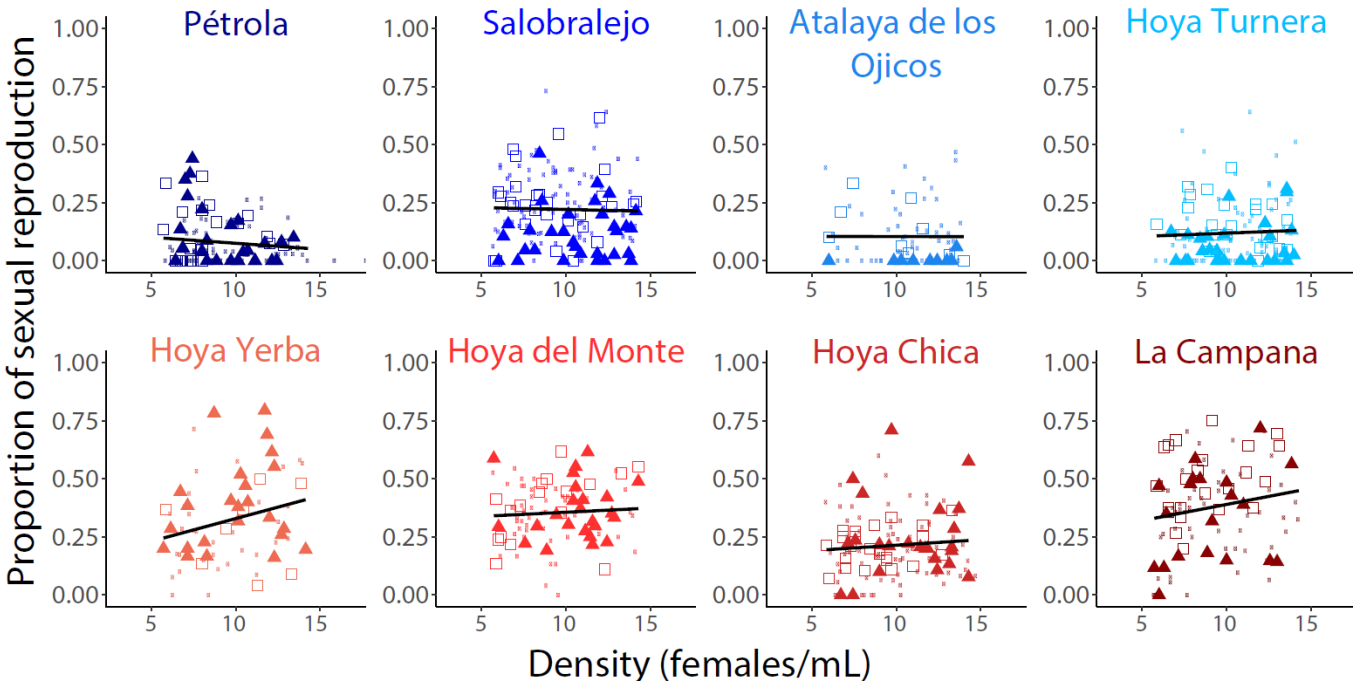


Figure 3.8. Effect of density on the proportion of sexual reproduction in *B. plicatilis* populations (triangles: F2 and F3; squares: F10 and F11; circles: F4 to F9). Lines in each plot are linear regression fits. In all cases, the correlation (unidirectional test) was not significant after Bonferroni's correction ($p > 0.00625$).

Discussion

The results in this chapter demonstrate 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 populations of *B. plicatilis*. This effect on the proportion of sexual reproduction after diapause confirms recent findings 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 Hino and Hirano 1977; Hagiwara et al. 2005). 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* (Gilbert 2002, 2003) and in *B. manjavacas* (Kamizono et al. 2017), a sibling species of *B. plicatilis*, among other monogonont species (Gilbert 1983; Schröder and 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 and 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 by which early and late generations of the same genotype respond differently to similar environmental (i.e., population density) conditions. Furthermore, this transgenerational effect is conceived 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.

The present study provides 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. This research, by studying clones from natural rotifer populations covering a gradient of environmental predictability (Franch-Gras et al. 2017a), provides 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 this bioassay design does not allow 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, results suggest that the trait does have adaptive value.

According to predictions for the present study, 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 to reach the maximum asymptotic value of the genotypically

determined PSR (Montero-Pau and 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 2.1), 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). A conjecture is 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 et al. 2017b) 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. Hydroperiod length should be considered 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 and 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, 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 and Gilbert 2004; Seudre et al. 2019). Genetic variation is necessary for the evolution of locally adapted traits. Therefore, the comparative analysis of habitat predictability performed in this thesis 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 and Hairston Jr. 1994; Ellner and Sasaki 1996).

In summary, the findings of the present chapter support the notion of a long-lasting adaptive nongenetic transgenerational effect occurring in rotifer populations. This chapter provides 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. This study demonstrates 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 these results may help in better designing the environmental settings in which possible mechanisms should be studied in the future (Chapters 1, 4 and 5).

4 Transgenerational gene expression profiles in relation to environmental predictability

Summary

A nongenetic 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 (Chapter 3). Notwithstanding, the molecular basis of this effect is still unknown (Chapter 1). In this chapter, 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. As expected, results in this chapter showed an increasing of expression of *edh* gene across generations in clones from those the more predictable ponds. This finding suggests a putative role of estradiol in the observed transgenerational effect. However, no differences in *meth* gene were detected across generations or in relation to environmental predictability. Nevertheless, alternative avenues for future research on the inherited gene regulation mechanism underlying this transgenerational effect are proposed.

Introduction

Cyclically parthenogenetic rotifers switch between reproductive modes in a density-dependent manner over their life cycle (Chapter 2). 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 and 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 and Gilbert 2004; Kamizono et al. 2017; Seudre et al. 2019; Chapter 3). This is a nongenetic 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 and 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). The extent of this transgenerational effect has been 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 (Chapter 2). 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. As it was reported in Chapter 3, *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, as it was explained in Chapter 1, the molecular mechanisms underlying the transgenerational effect on sexual reproduction found in rotifers are unknown. This inhibition of sex in early generations after diapause could be mediated by cytoplasmatic cellular components such as nutrients, metabolites or hormones (see Chapter 5) or, alternatively, by any kind of transgenerational epigenetic inheritance

mechanism (e.g., DNA methylation, histone modifications, or noncoding RNAs; Jablonka and Raz 2009). As it was introduced in Chapter 1, DNA methylation is considered a common epigenetic signalling mechanism for inhibiting gene expression (Bird and Wolffe 1999; Bird 2002) in many generations (Angers et al. 2010) and that has been observed in many species (Adams 1996; Field et al. 2004). Moreover, DNA methylation is involved in the expression control of genes related to hormones in organisms with complex life cycles. Although, little information is available to date on methylation patterns in rotifers. 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, the starting hypothesis of the research addressed in the present chapter was 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 (Gallardo et al. 1997, 1999, 2000a, 2000b; Radix et al. 2002; Snell and 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 thesis, 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. The expectation is that the expression level of *edh* would increase along with generations in clones from the populations inhabiting the more predictable ponds, whereas it would 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* (Chapter 3). Assuming that *meth* is a methylation maintenance gene for silencing the expression of other genes related to sexual reproduction, in this study, it was hypothesized that its expression level

would remain low in clones from populations inhabiting unpredictable ponds and that it would 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 the experiment described in Chapter 3, in which the transgenerational effect on the proportion of sexual reproduction in *B. plicatilis* populations was assessed. In short, in that experiment, populations inhabiting four saline ponds in Eastern Spain, which comprise a gradient spanning from low to highly predictable lengths of the growing season were tested (Table 4.1). The index of environmental predictability used is based on presence or absence of water, considering the perception of environmental fluctuation by *B. plicatilis* individuals according to the biological information available for the studied ponds (see Franch-Gras et al. 2017a and Chapter 2 for further details).

Table 4.1. Studied natural populations and features of the ponds where they inhabit: mean water-surface area (m^2), salinity (g/L), degree of environmental predictability, and hydroperiod length (adapted from Franch-Gras et al. 2017b).

Natural populations	Acronym	Mean water-surface 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	1,060	2.84-16.5	0.34	0.23
Hoya Chica	HYC	32,000	10.79	0.12	0.51

Diapausing eggs used to perform this experiment were produced by the same laboratory multiclonal populations described in Chapter 3 (Figure 3.2). In summary, ten 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 ten P-clones. Diapausing eggs from these multiclonal populations of approximately the same age and produced under controlled conditions were harvested and stored in saline water (60 g/L 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 and Hino 1989; Martínez-Ruiz and 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 with the microalgae *Tetraselmis suecica* as a food source at 250,000 cells m/L (ca. 33 mg C/L) grown in a chemostat at high cell concentration to maintain their quality (Figure A.3 in Appendix 3). 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 (Figure 4.1). When these females were sexual or died, they were substituted by offspring of the same generation from another replicate. 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 (Figure 3.3 and Figure 4.1). 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 (Figure 4.1). The concentration of *T. suecica* in the culture medium was 500,000 cells m/L except for four clones where it was 250,000 cells m/L (further details in Chapter 3). 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 saline water to remove microalgal cells while retaining rotifers.

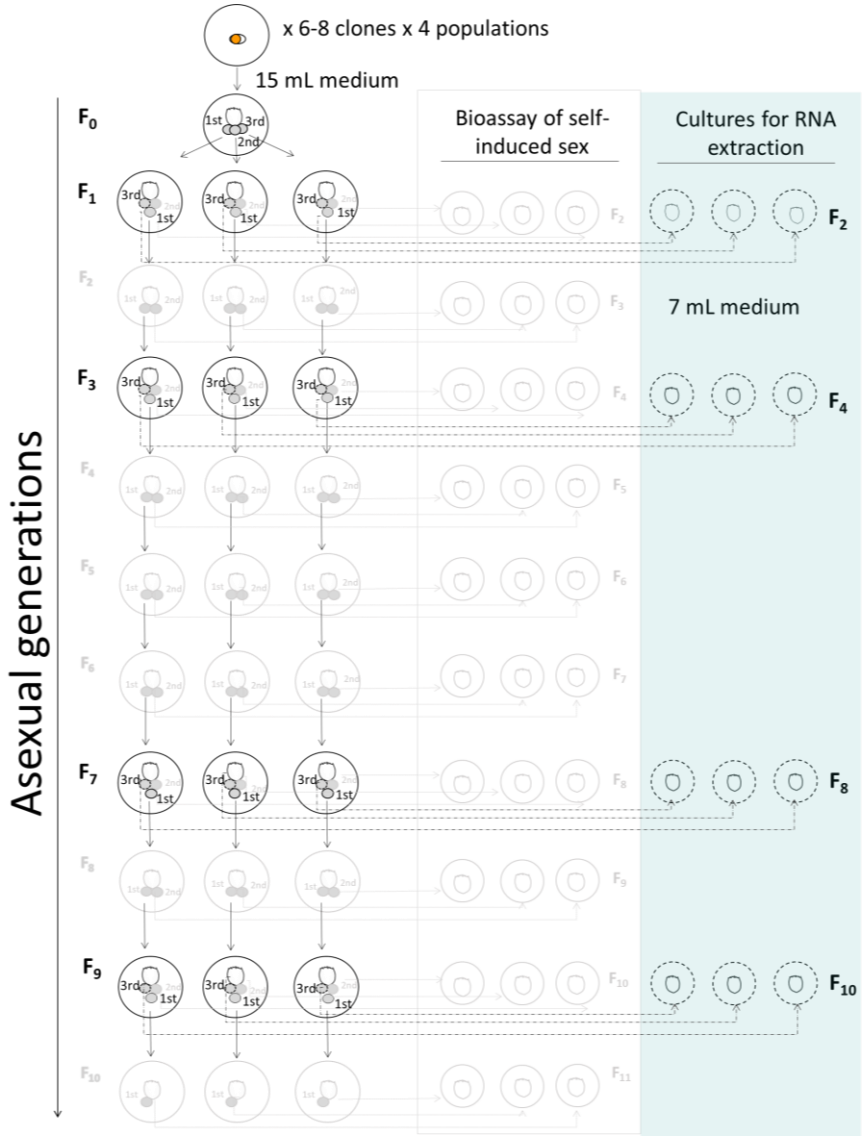


Figure 4.1. Schematic diagram showing the experimental design performed for each *B. plicatilis* clone. RNA samples were obtained for 6–8 clones of each of the four studied populations as a part of the previous study carried out in Chapter 3.

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 Directzol™-96 RNA (Zymo Research). RNA samples were retrotranscribed to cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen) and poly 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 A260/280 lower than 1.8 (Fleige and Pfaffl 2006) and a concentration lower than 10 ng µ/L 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 during cDNA amplification (Sandhu and Acharya 2005). 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, 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 were analysed. 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 4.2). 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 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 µ/L) was normalized to the *btf3* cDNA copy µ/L as previously described (Cucuzza et al. 2017). In detail, each qPCR analysis was performed 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 4.2. 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 µ/L).

Table 4.2. 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			

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 µ/L values obtained for both standards and samples were converted in gene copy µ/L 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 µ/L and the size (bp) of each amplicon (shown in Table 4.2), the number of gene copy µ/L was calculated. According to Bustin et al. (2009), the limits of quantification per each gene were 1700 copy µ/L for *btf3*, 109 copy µ/L for *edh*, and 158 copy µ/L 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 Generalised Linear Mixed Models (GLMMs). Gaussian distribution of

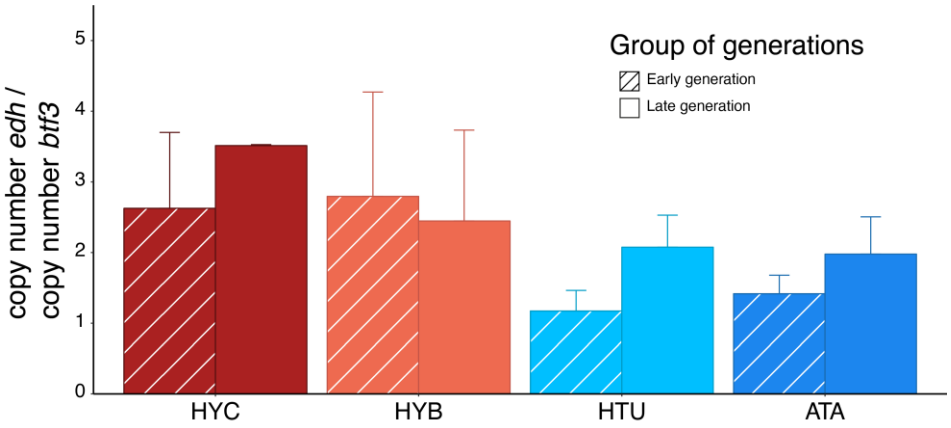
errors and identity link function were used (Crawley 2013). The formulation of GLMMs were identical for both genes and included generation order and predictability degree of the origin locations, as well as their interactions, as fixed effect explanatory variables. F-clones were treated as levels of 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. Nonsignificant 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, 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; data from Chapter 3) 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

The results showed decreasing expression levels of *edh* with increasing environmental predictability of the origin ponds of the clones and populations studied (Figure 4.2A), this effect was statistically significant (Table 4.3). Interestingly, in those results also showed an increase in the expression level of *edh* when comparing early and late generations in the populations of the most predictable ponds (Figure 4.2A), the interaction effect between generation order and predictability was significant too (Table 4.3). 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* (Figure 4.2B) and none of the effects tested by GLMM were significant (Table 4.3). 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 $\chi 2 = 3.420$, $df = 2$, $p = 0.181$).

(A)



(B)

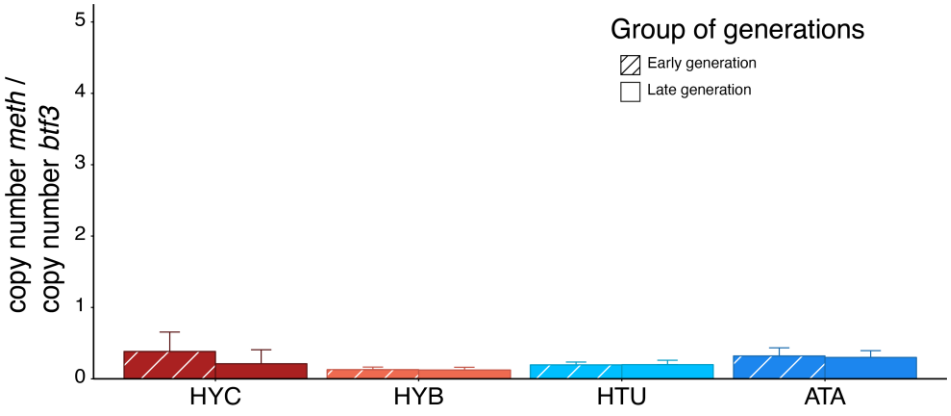


Figure 4.2. Comparison of the expression level of **A** the *edh* gene and **B** the *meth* gene between two groups of generations (*early*: F2 and F4; *late*: F8 and F10) in populations of *B. plicatilis* from four ponds (HYC, HYB, HTU and ATA). Environmental predictability is coded using a colour gradient from darkest red (more unpredictable ponds) to darkest blue (more predictable ponds).

Table 4.3. Effect of generation order, habitat predictability and their interaction on the logarithmic copy number in the four studied populations of rotifer *B. plicatilis* for *edh* and *meth* genes.

Effect	***Df***	***edh***		***meth***	
		F	***p***	***F***	***P***
Generation	1	2.460	0.117	3.420	0.064
Predictability	1	7.100	0.008	0.011	0.916
Generation x Predictability	1	4.203	0.040	1.003	0.317

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 (Figure 4.3; $r = 0.62$, $p = 0.019$).

Discussion

Many studies have shown the inhibition of sexual reproduction across the early generations following diapause in rotifers (Hino and Hirano 1977; Gilbert 2002, 2003; Schröder and Gilbert 2004; Kamizono et al. 2017; Seudre et al. 2019). In Chapter 3, the contribution of environmental predictability to this transgenerational effect has been demonstrated and its importance in the adaptation of rotifers to fluctuating environments has been highlighted. Nevertheless, little was known about the underlying molecular mechanisms (Chapter 1).

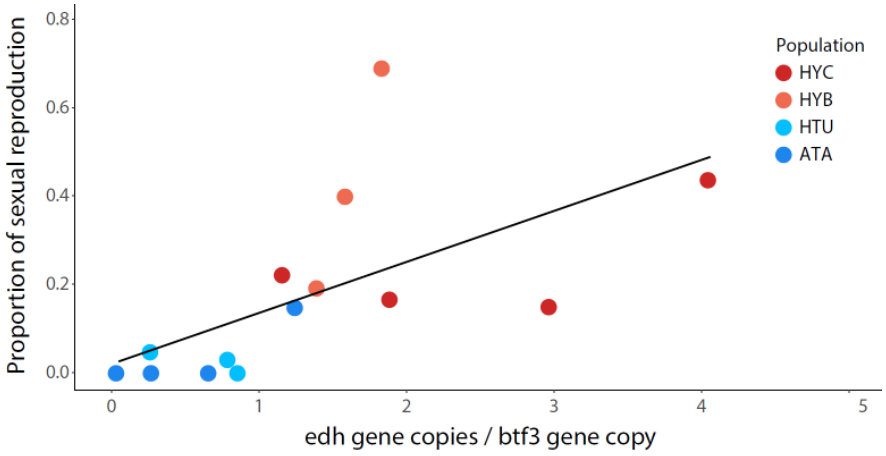


Figure 4.3. Relationship between the proportion of sexual reproduction (data from Chapter 3) and *edh* copy number from the same set of *B. plicatilis* clones from each studied population. Environmental predictability is coded using a colour gradient from darkest red (rotifer populations inhabiting more 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.

Therefore, the main aim in the present chapter was addressing the expression profile of two genes that could be involved in the nongenetic inheritance of this transgenerational effect. In line with initial expectations, a significant increase in expression level of *edh* was observed 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 and Adamski 2009), the low expression of the gene would be expected to result in a reduced level of estradiol. The synthesis of sex steroid hormones is involved in reproductive processes in aquatic invertebrates (Köhler et al. 2007; Lafont and 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 the finding that the proportion of sexual reproduction measured in Chapter

3 is significantly and positively correlated with *edh* expression level quantified in the present chapter 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 (Chapter 3). Low expression levels of 17-*β*-dehydrogenase 12, 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). As it was described in Chapter 2, 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, 2017b). 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 length of 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 study, DNA methylation was proposed that 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, this study 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, changes in the expression level of *meth* across generations were not found, 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 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) cannot be ruled out.

Results in this chapter have been an attempt to shed light on the molecular mechanisms implied in the nongenetic transgenerational effect of inhibition of sexual reproduction in *B. plicatilis*. In the present study a possible role of the 17-*β*-estradiol hormone mediated by the expression of *edh* encoding for the 17-*β*-hydroxysteroid dehydrogenase 12 in *B. plicatilis* has been documented. 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 the timing of sexual reproduction and the investment in it by quantifying 17-*β*-estradiol levels or the increase in *edh* gene expression can be tracked. Despite the above, it remains to be found a mechanism that explains the transgenerational effect. Results obtained here 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 the 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, micro-RNAs, or by other cytoplasmatic factors that affect gene expression yet to be determined, are the subject of research in Chapter 5.

5 Effect of laying order on offspring reproductive mode in relation to the nongenetic trans-generational inhibition of sex in *Brachionus plicatilis*

Summary

A nongenetic transgenerational inhibition of sexual reproduction in *Brachionus plicatilis* was confirmed in previous chapters (Chapters 3 and 4). Nevertheless, the underlying mechanism of this transgenerational effect remains unclear. One possibility is that genome-associated processes, such as DNA methylation, might explain the effect. Notwithstanding, no evidence of methylation was found when tracking the expression of a DNA methyltransferase gene across generations in different populations of *B. plicatilis* (Chapter 4). Alternatively, it has been proposed that this effect could be mediated by cytoplasmatic cellular components (e.g., nutrients, metabolites, or hormones), which may be passed from mothers to daughters, gradually decreasing in concentration

and thereby diminishing their inhibitory effect transgenerationally. This would imply an increasing sexual response across generations. Worth mentioning, such a mechanism would also work among the whole offspring within a given generation, leading to a reduction of the concentration of the metabolite responsible for the inhibitory effect on sexual reproduction with each subsequent egg-laying (i.e., with laying order). If so, this would imply an increasing sexual response with laying order, thereby increasing sexual response in later-laid offspring within the same generation. In this chapter this prediction was tested by quantifying the proportion of sexual reproduction (PSR) in the offspring of rotifer females from the first and tenth generations after hatching from the diapausing egg in several clones of *B. plicatilis*. Results showed that sexual reproduction was indeed higher in the tenth generation compared to the first, reconfirming the nongenetic transgenerational inhibitory effect. However, no differences in the sexual response were observed across the laying order within either generation. These findings do not support the hypothesis of a control mechanism for this transgenerational inhibitory effect mediated by cytoplasmatic components being transferred from mother to offspring. Alternative mechanisms are discussed as potential subjects for further research.

Introduction

A nongenetic transgenerational inhibitory effect on the sexual response to population density after leaving diapause has been recently observed in *Brachionus plicatilis* (Seudre et al. 2019; see Chapters 3 and 4 in this thesis), as also before in other species of monogonont rotifers (Gilbert 2002, 2003, 2017; Gilbert and Schröder 2004). This transgenerational effect implies that, regardless of population density, the ability of females to initiate sexual reproduction after diapausing egg hatching is low, then increasing over generations. Nevertheless, the molecular mechanisms to explain this transgenerational inhibitory effect are not known. This inhibition could be mediated by a transgenerational epigenetic inheritance mechanism as genome-associated processes (DNA methylation or histone modifications) or by the contribution of cytoplasmatic cellular components such as nutrients, metabolites or hormones supplied by mothers to their progeny in the process called maternal provisioning (Jablonka and Raz 2009; Adrian-Kalchhauser et al. 2020). While DNA methylation seems to be a plausible mechanism, as it silences the expression of genes in several generations (Bird 2002; Angers et al. 2010), previous attempts (see Chapter 4) have failed to detect differential methyltransferase gene expression across generations in *B. plicatilis*.

Alternatively, cytoplasmatic components may play a role in the transgenerational inhibitory effect of sexual reproduction in rotifers. It is well established that endogenous inhibitory factors can influence oocyte development, causing them to produce specific morphs over others –e.g., variation in presence or size (Schröder and Gilbert 2009) or type of reproductive females (Gilbert 2003; but see Gilbert 2017 for review). Along the same lines, Gilbert (2002, 2003, 2017) has proposed that the transgenerational effect on sexual reproduction may be regulated by an endogenous control mechanism, possibly involving some sort of maternally provided cytoplasmatic factor. This yet-to-be-determined factor would be present in diapausing eggs inhibiting sexual reproduction in early generations, with its concentration –and therefore its inhibitory effect– diminishing over successive generations (Gilbert 2002). Gilbert (2017) has proposed that this hypothetical factor may function in one of two ways: (1) by blocking the reception of the protein responsible for inducing sexual reproduction (the so-called MIP; see Chapter 2) or, alternatively, (2) by inhibiting the production of any steroid hormone involved in sexual reproduction. In either case, this endogenous factor would promote asexual reproduction. It is worth mentioning that the concentration of this hypothetical factor transferred to the next generation would likely depend on the number of offspring a female produces during her reproductive period, which could act as a confounding effect. If a cytoplasmatic factor that inhibits the production

of sexual females is indeed transferred transgenerationally from mothers to daughters, it would also impact the oocytes from the offspring within a generation. Consequently, the sexual response to density would increase both across generations and within a generation as the concentration of the inhibitory factor decreases. Different outcomes could be expected depending on both the fecundity (i.e., the total number of offspring) of the stem female (i.e., the female hatching from the diapausing egg) and the distribution of the potential cytoplasmatic factor among her offspring, whether or not it is equally allocated, as the available amount of the factor could decrease progressively over the reproductive period of the mother and therefore with laying order (illustrated in the four scenarios in Box 5.1). If the cytoplasmatic factor is evenly distributed among the offspring (scenarios A and B in Box 5.1), its concentration per daughter would decrease with the number of offspring produced. This would mean –assuming an equal initial amount of the cytoplasmatic factor in stem females– a higher inhibition of sexual reproduction and, thus, a lower number of sexual daughters (i.e., lower proportion of sexual reproduction, PSR) when the fecundity of the mother is low compared to when it is high. Alternatively, the cytoplasmatic factor may be distributed unequally, with the highest concentration transferred to the first daughter and decreasing with each subsequent offspring, i.e., with laying order (scenarios C and D in Box 5.1). This case is more plausible than the even distribution of the cytoplasmatic

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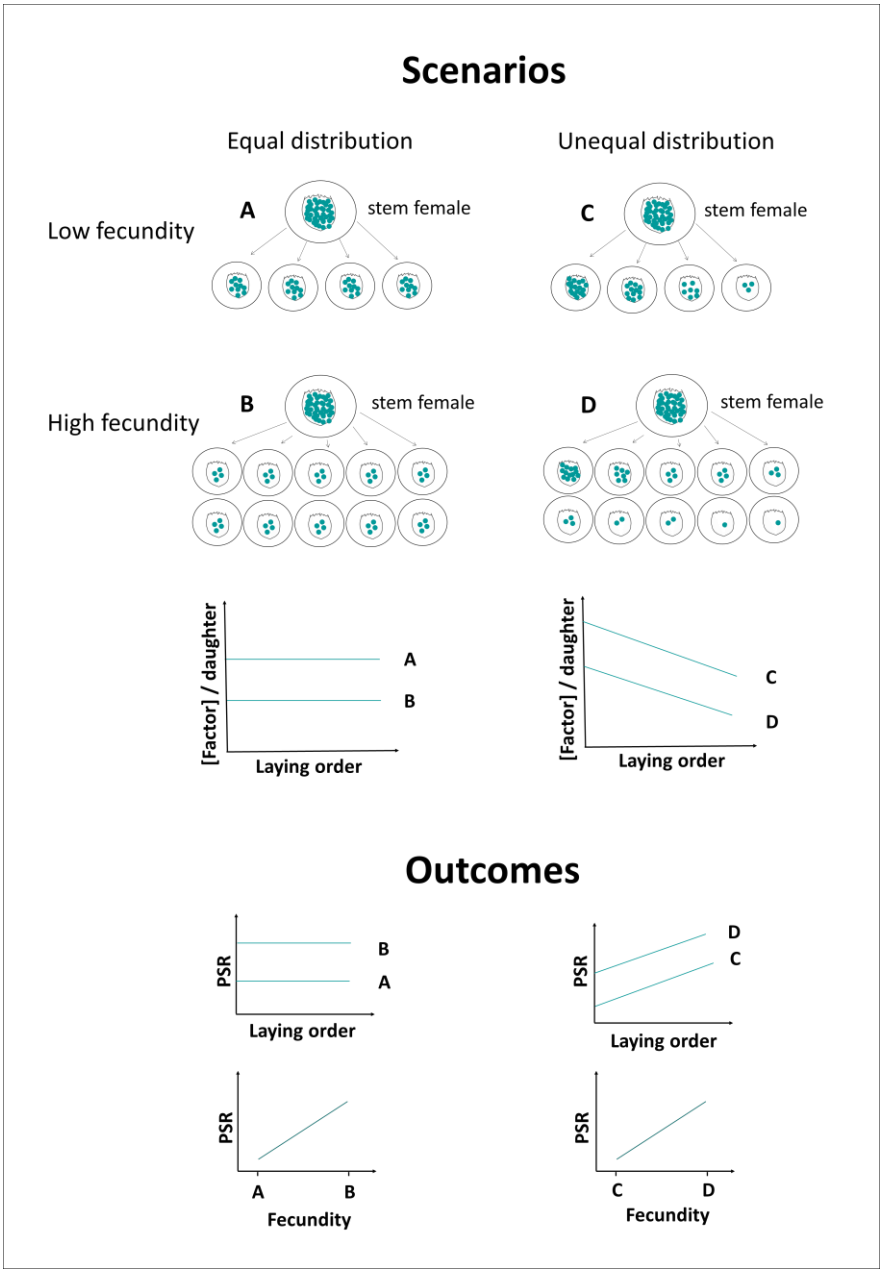
Box 5.1. Hypothetical outcomes for sexual reproduction patterns in the offspring of stem females are presented based on their laying order and maternal fecundity, assuming the transfer of a cytoplasmatic endogenous factor from mother to daughters that inhibits sexual reproduction. The four scenarios (A-D) illustrate variations based on two key factors: the mode of distribution of the inhibitory factor among the offspring (equal or unequal) and the fecundity of the mother (low or high). Green dots represent the idealized amount of a putative endogenous cytoplasmatic factor. In separate columns, depending on the hypothetical distribution pattern, qualitative expectations are depicted for (1) the factor concentration per female by laying order in the offspring of the stem female, (2) the proportion of sexual reproduction ratio (PSR) by laying order, and (3) the expected relationship between the PSR and maternal fecundity, with each displayed in biplots from top to bottom.

Effect of laying order on offspring reproductive mode across generations



factor, since there may be some uncertainty regarding the number of daughters that a female can produce during her lifetime. In both scenarios, C and D, the PSR would increase with laying order as the concentration of the inhibitory factor declines. As with the even distribution case, greater fecundity would lead to higher PSR.

In this chapter, the possible outcomes outlined above are examined to distinguish among the different hypothetical scenarios. The goal was to test whether the transgenerational inhibitory effect on sexual reproduction, reported in Chapter 3, could be mediated by a cytoplasmatic cellular factor, as suggested by Gilbert (2002, 2003, 2017). To this end, the PSR was estimated across laying order in the offspring of females from the first and tenth generation of *B. plicatilis* stem females. If a cytoplasmatic factor underlies the transgenerational effect in *B. plicatilis*, the expectation is that PSR in the offspring will increase with the fecundity of mothers within a given generation. Furthermore, depending on the distribution of the cytoplasmatic factor among the offspring of the same generation –either equally among all offspring or with a decreasing allocation as laying order increases– it would be observed either a uniform PSR across laying order or an increase in PSR with laying order, respectively, as depicted in the scenarios outlined in Box 5.1.

Material and methods

Origin population and experimental clones

The potential effect of laying order underlying the inhibitory effect on sexual reproduction in *B. plicatilis* was studied in several clones from Hoya Turnera (HTU), a pond characterised as predictable with respect to the inter-annual variation of the length of its hydroperiod (Franch-Gras et al. 2017a; more details in Chapter 2). This population was chosen because the inhibitory effect on sexual reproduction in the first generations after hatching from the diapausing egg had previously been observed in it (see Chapter 3).

As in previous chapters, diapausing eggs from this population were sampled from the sediment egg bank of the pond in order to establish a set of parental clones (hereafter, P-clones). These clones were maintained individually under standard conditions (25 °C, 12 g/L salinity and 35 µmol quanta/m^2 s; see Chapter 3). Three multiclonal laboratory populations were created by mixing five females from each of 10 P-clones, allowed to grow and produce new cohorts of diapausing eggs under controlled, homogenizing laboratory conditions (see Figure 3.2, Chapter 3). Prior to experiments, these diapausing eggs were collected, pooled, and stored in saline water (60 g/L) at 4 ^{o}C for at least one month (Hagiwara and Hino 1989; Martínez-Ruiz and García-Roger 2015). After this obligate period of diapause, diapausing eggs were incubated under

standard conditions to induce hatching (25 °C, 6 g/L salinity and 150-170 µmol quanta/m^2 s; García-Roger et al. 2005, 2006; Martínez-Ruiz and García-Roger 2015; see Chapter 3). From these hatchlings, eight stem females (F0) were used as experimental clones (hereafter, F-clones). Each stem female was individually cultured in 15 mL of fresh culture medium of the microalgae *Tetraselmis suecica* at 250,000 cells/mL (c. 33 mg C/L) in 60-mm diameter plastic Petri dishes and served to establish a clonal lineage (as in Chapters 3 and 4). Each lineage was used as a replicate in a new transgenerational common garden experiment (see below and Chapter 2).

Propagation of experimental lineages

Subsequent generations of each lineage –up to a maximum of 10 generations (F10)– were repeatedly initiated by transferring the first daughter of the previous generation to fresh culture medium (Figure 5.1; Chapters 3 and 4). Whenever possible, additional females from each generation were maintained under the same conditions as the main lineage. In case the female of the main lineage died or was sexual, replacements were taken from these additional females.

In parallel with the propagation of generations, the offspring of the F0 and F10 generations of each lineage were followed to study the sexual response to density across laying order (see below). These two

generations were selected based on findings from Chapters 3 and 4: the F0 was the generation in which the nongenetic transgenerational inhibitory effect was confirmed, as indicated by the low PSR values observed, whereas this effect was no longer present in the F10 generation.

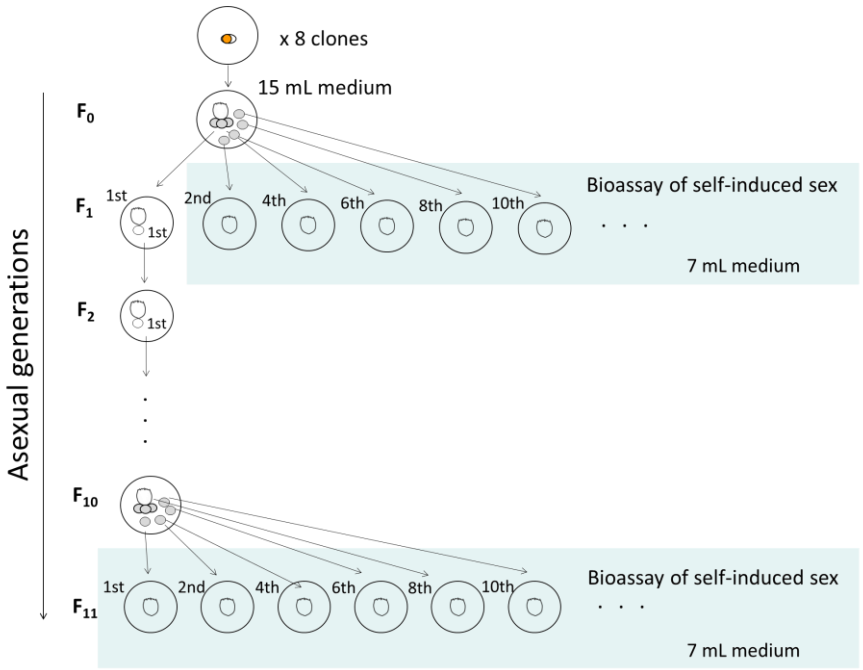


Figure 5.1. Schematic diagram of the experimental design used to study the effect of laying order on the proportion of sexual reproduction (PSR) in eight clones of *B. plicatilis* in two different generations (F0 vs F10). Details are provided on both the propagation of generations and the bioassays of self-induced sex used to estimate PSR.

Bioassays of self-induced sexual reproduction

Bioassays where sexual reproduction was induced by the growing population itself initiated from a female (see Chapter 3) were carried out to measure PSR across laying order in the offspring of the F0 (that is in the F1) and F10 (that is in the F11) generations of each lineage under standardized sex inducing conditions. The even-numbered daughters in the laying sequence of F0 and F10 females were used in the bioassays (Figure 5.1), along with the first daughter from F10. It is important to note that, in each lineage, the first daughter from both F0 and the subsequent generations was used to propagate the lineage (see above).

To conduct the bioassays, F1 and F11 neonate females from each lineage were placed individually in 60-mm plastic Petri dishes containing 7 mL of saline water at 12 g/L with 500,000 cells m/L of *T. suecica* cultures previously grown in a chemostat (see Chapter 3). At 24-h intervals, and continuing until their death, the mothers (F0 and F10 females) were individually transferred to fresh culture medium (15 mL containing *T. suecica* at 250,000 cells/mL) to avoid the accumulation of the sex-inducing protein (MIP; see Chapter 3) in the culture. This procedure prevented the induction of sexual reproduction in the daughters before they were isolated for the bioassays. In each bioassay, each daughter was allowed to proliferate asexually, and the culture was monitored daily until the population reached 6-10 females/mL –a density high enough to

induce sexual reproduction in *B. plicatilis* (Carmona et al. 1993; Stelzer and Snell 2003; García-Roger et al. 2009). At that point, the cultures were fixed with Lugol's solution (4% final concentration), and the females (both ovigerous and juveniles) were counted under a stereomicroscope. Ovigerous females can be differentiated into sexual or asexual females based on the size and morphology of their eggs (Carmona et al. 1995; Figure 3.4; Chapter 3). Finally, PSR in each bioassay was estimated by the proportion of sexual females relative to the total number of ovigerous females.

The experimental approach followed in these bioassays was similar to that of Gilbert (2002) and Gilbert and Schröder (2004) to standardise the final rotifer abundance in each bioassay, thereby ensuring a similar concentration of MIP across clones and generations (for more details, see Chapter 3). A total of 159 bioassays was performed as a result of combining 8 F-clones x 2 generations (F1 and F11) x 2-14 daughters according to their order of laying.

Data analysis

Raw data were filtered by the final density reached in the bioassay cultures to (1) obtain reliable PSR estimates from counts with a sufficient number of females, and (2) ensure consistent sex-induction conditions were across clones and generations. In this way, bioassays in which the

final female density was out of the range of 6-10 females/mL were excluded, resulting in the removal of 86 out of 159 bioassays. Prior to further analyses, an outlier scan was carried out, and atypical data were removed. Then, PSR (i.e., the counts of sexual versus asexual females) was analysed using a Generalised Linear Model (GLM) assuming a binomial distribution of errors and logit as a link function (Nelder and Wedderburn 1972). This model included generation, laying order, and female density as explanatory variables, along with their interactions. Statistical significance of each variable was assessed at a threshold of α = 0.05. The GLM was implemented by means of *glm* and *anova* functions from the stats package in R 4.1.0 statistical software (R Core Team 2021). The Akaike Information Criterion (AIC) was also examined, using the *AIC* function from the same R package, to verify that the GLM performed a better fit to the dataset without outliers. Finally, Pearson's correlation coefficient between fecundity in F0 (number of daughters produced by each stem female) and PSR in the offspring was calculated using the *cor.test* function from the stats package in R.

Results

The distribution of PSR values for the two generations studied, F1 and F11 is shown in Figures 5.2 and 5.3. Values of PSR in clones from F11 were higher than those in F1, in agreement with previous findings (see Chapter 3).

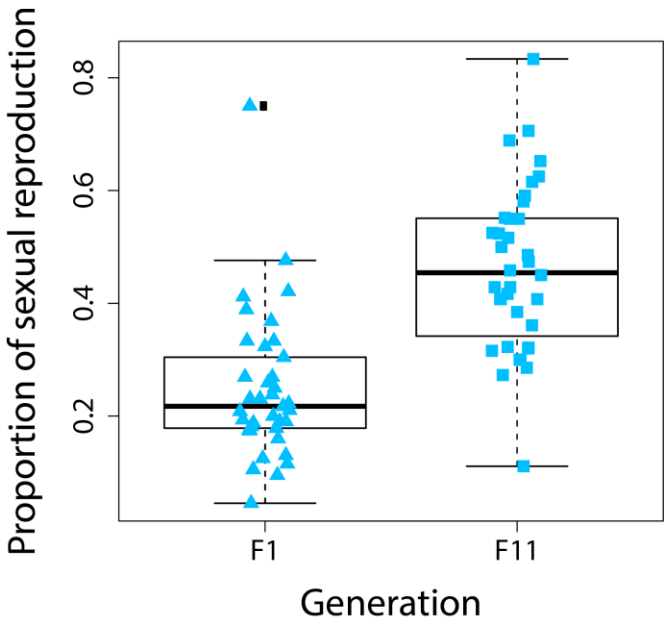


Figure 5.2. Box-plots and jittered data for the proportion of sexual reproduction (PSR) of *B. plicatilis* from the bioassays conducted for generations F1 and F11 of the eight clonal lineages studied. Outlier is indicated as black square.

Once the data were filtered to ensure comparable density ranges and the outliers were removed (Figure 5.2), results from the 72 bioassays showed no significant effect of laying order on PSR, neither in the offspring of the stem females nor in offspring of the tenth-generation females (Figure 5.3, Table 5.1). In contrast, the difference in PSR between F1 and F11 generations was statistically significant (Table 5.1), with higher PSR in the latter generations than in the first ones (Figures 5.2 and 5.3).

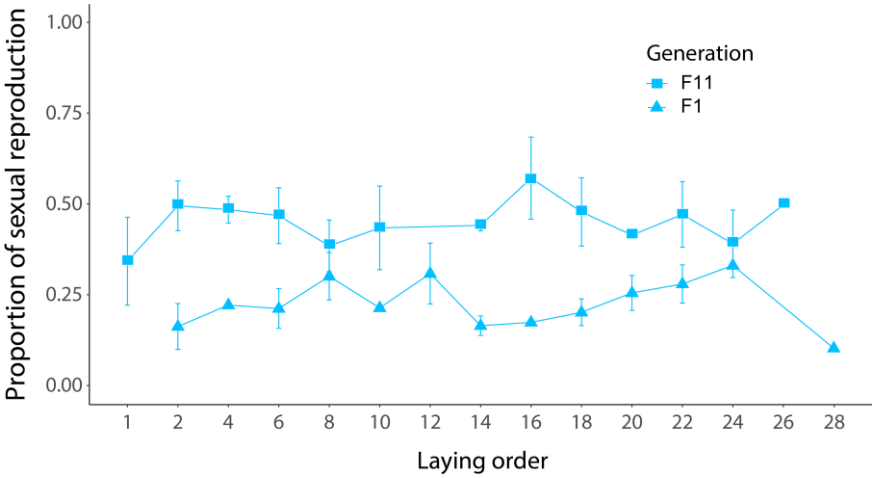


Figure 5.3. Proportion of sexual reproduction (PSR) across the laying order corresponding to first (F1) and eleventh (F11) generation in *B. plicatilis*. Values are averages for eight clones with vertical bars representing the standard error.

The interaction between generation and laying order was not statistically significant (Table 5.1). Overall, these results support that PSR does not show any change in sexual response to density along laying order in any generation studied. Although F-clones exhibited genetic variability in PSR among them within each generation, the sexual response to laying order was similar within in each generation (Figures A.4.1A and A.4.1B). The interaction between total female density and laying order was also not significant (Table 5.1). The GLM model performed better on the dataset without outliers (AIC = 368) than on the full dataset (AIC = 374).

Table 5.1. Summary of effects of the generalised linear model (GLM) performed on the proportion of sexual reproduction in eight clones of the rotifer *B. plicatilis*.

Effect	***Df***	***Deviance***	***P***
Laying order	1	0.60	0.437
Generation	1	104.77	< 0.001
Density	1	2.08	0.154
Laying order x Generation	1	0.28	0.595
Laying order x Density	1	1.76	0.185
Generation x Density	1	0.21	0.649
Laying order x Generation x Density	1	0.387	0.534

Contrary to expectation, no relationship was found between the PSR of the offspring and the fecundity of the stem females (Figure 5.4; $r = 0.057$; $df = 6$; $p = 0.893$).

Discussion

This study provides the first approach to exploring the potential effects of a hypothetical cytoplasmatic endogenous factor accounting for the inhibitory effect observed on rotifer sexual reproduction in the first generations of females after hatching from the diapausing eggs.

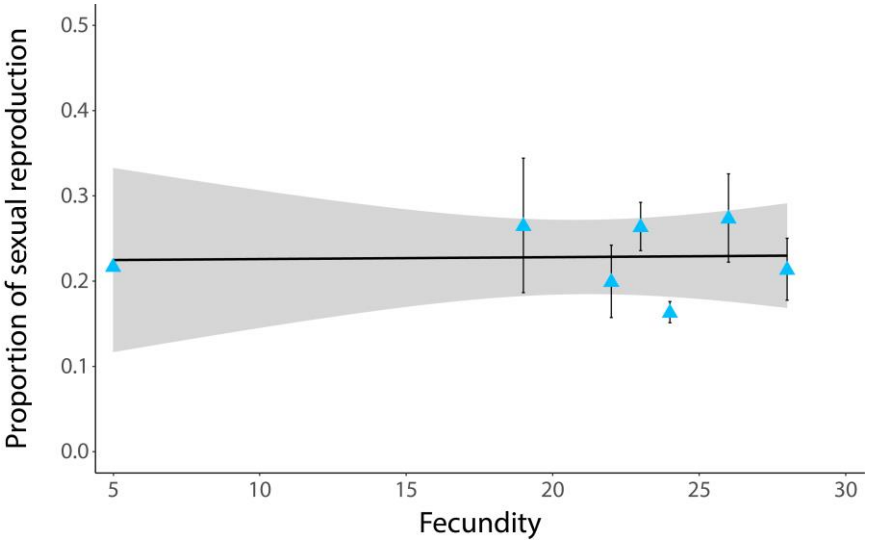


Figure 5.4. Relationship between the proportion of sexual reproduction (PSR) and the fecundity in the stem females of the eight clones of *B. plicatilis* studied. Vertical bars are the standard error. The trend line represents the least squares linear regression fitted to the data.

Specifically, here it was tested the prediction –under this hypothesis of the transgenerational effect mediated by a cytoplasmatic factor– that as the factor is transferred from mothers to daughters, the proportion of sexual reproduction (PSR) in the offspring should increase with both the fecundity of the stem female and laying order. In contrasts with these expectations, no effect of laying order or fecundity on PSR in F1 was detected. Regarding laying order, these findings were consistent with preliminary observations obtained in Chapter 3, where no significant differences in PSR were found among the first three females of the same

clonal lineage within a generation (see Chapter 3 and Figure A.4 in Appendix 4). Therefore, results from the present chapter do not support the hypothesis that a progressive reduction in the allocation of a potential inhibitory cytoplasmatic factor among the offspring explains the nongenetic transgenerational inhibitory effect on sexual response. Additionally, no effect of laying order was found in F11 either, although, if present, any such might be attenuated by the passage of generations. Furthermore, no differences in PSR related to the fecundity of stem females were found in this chapter. This finding also does not support the idea that the transgenerational effect could be mediated by the transfer of cytoplasmatic components from mothers to daughters. Notwithstanding, this conclusion should be interpreted with caution, since the experimental clones used as replicates had different fecundities, and it cannot be ruled out that there may be also genetic differences in the amount of the cytoplasmatic factor that would be transferred from the stem female to her offspring, if this was indeed the underlying mechanism. Differences in PSR between the two generations studied –despite the fact that the inducing stimulus (the population density) was similar in all bioassays– confirm the nongenetic transgenerational inhibitory effect on sexual reproduction in *B. plicatilis* formerly reported by Seudre et al. (2019) and in previous chapters of this thesis (Chapters 3 and 4; Figure 3.5, Figure 3.7, and Figure 4.2A). Furthermore, the sexual response to density was more variable between

the clones tested in the F11 than in the F1, suggesting a potential genotypic control on the degree of the inhibitory effect (i.e., affecting more or fewer generations). Within-clone replicate trials would be needed to test whether the different fecundities of the clones tested could mediate the greater or lesser number of generations in which the inhibitory effect is expressed. It is important to note that although the trait studied –the sexual response to population density– is clearly plastic, as a genotype can express one phenotype or another (sexual reproduction is either induced or not) in response to the same inducing stimulus depending on the generation, there could be genetic differences in the degree of plasticity of the trait. In alignment with other studies on rotifers (Schröder and Gilbert 2004; Seudre et al. 2019; Chapters 3 and 4), this chapter also demonstrates the existence of genetic variability among clones within the same population (Figure A.5 in Appendix 5), which may fuel the potential for evolution of locally adapted traits.

Overall, the results found here are not compatible with the hypothesis of a mechanism based on the mother-daughter transfer of an endogenous cytoplasmatic factor that inhibits the sexual response to population density stimulus. Alternative explanatory mechanisms, to the one based on maternal provisioning, for this transgenerational effect are genome-associated processes, such as DNA methylation and histone modifications (Jablonka and Raz 2009) silencing the expression of sexual reproduction-related genes. Regarding methylation, it is known that it can affect more

generations than cytoplasmatic factors (Anastasiadi et al. 2021) and, therefore, might be a candidate mechanism to explain the observed transgenerational effect on sexual response in *B. plicatilis* and other rotifer species. Although results from Chapter 4 showed no difference in the expression of the DNA methyltransferase (*dmnt2*) neither between generations nor among populations from habitats with different degree of environmental predictability, some form of methylation mechanism cannot be ruled out. Diapausing eggs produced under different regimes of environmental unpredictability have been shown to exhibit different levels of methyltransferase activity (Tarazona et al. 2020). Consequently, spontaneous demethylation processes may facilitate the reversal of gene silencing and unlock the sexual response to population density (see also Chapter 4).

Histone modifications are also known to be involved in the regulation of gene expression and in the differential expression of life history traits in the offspring (Kouzarides 2007; Skinner et al. 2010; Lee et al. 2020). Modifications of histones tails via mechanisms such as acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination or proline isomerization, could be involved in gene expression regulation (Peterson and Laniel 2004; Kouzarides 2007). Recently, histone modifications have been discovered in several rotifer species –*Brachionus manjavacas* (Gribble and Mark Welch 2017), *Brachionus koreanus* (Lee et al. 2020), *Brachionus calyciflorus* and

Brachionus fernandoi (Paraskevopoulou et al. 2020)– being implicated in the changes in gene expression related to ageing and to the response to environmental stressors, such as low pH and heat tolerance.

While direct transcriptional evidence for histone modification as a regulator of diapause-related traits in rotifers is currently lacking, previous studies have found that histone methylation and acetylation influence diapause stages in a wide range of insects (Reynolds 2017). For instance, it has been reported the role of histone methylation in the photoperiodic diapause induction in the larvae of the drosophilid fly *Chymomyza costata* (Poupardin et al. 2015) and in the adults of both fruit fly *Drosophila melanogaster* (DiVito Evans et al. 2023) and the mosquito *Culex pipiens* (Wei et al. 2023). There are also recent findings on the important role of histone acetylation for pupal diapause in the fly *Sarcophaga bullata* (Reynolds et al. 2016) and the cotton bollworm, *Helicoverpa armigera* (Lu et al. 2023). Therefore, given the evolutionary conservation of diapause in invertebrates (Alekseev et al. 2007) and the growing evidence that these two phylogenetically distant groups –rotifers and insects– share physiological and molecular mechanisms underlying diapause (e.g., García-Roger et al. 2019), it is likely that a role for histone modification in the regulation of diapause in rotifers will be revealed in the near future. It is still even more likely because diapause-induced transgenerational effects in line to the one reported here (Chapter 3)

occur in some of these insect species (i.e., *D. melanogaster*, Di Vito Evans et al. 2023; and *S. bullata,* Reynolds et al. 2013).

The results of the study conducted in the present chapter do not allow to ascertain the mechanism underlying the transgenerational effect on sexual reproduction in *B. plicatilis*. However, in the absence of evidence for maternal provisioning of any cytoplasmatic factor to daughters, DNA-based epigenetic regulatory mechanisms –such as DNA methylation and histone modifications– emerge as the best-placed candidates. Future research into these mechanisms will hopefully elucidate the molecular basis of this transgenerational effect.

6 General discussion and conclusions

Evolution studies the processes that generate biological diversity on Earth, while ecology investigates the relationships between organisms and the environment. These two disciplines intersect in the field of evolutionary ecology when it comes to understanding how organisms adapt to changing environmental conditions. Darwin initiated this line of thought before ecology became a science, by recognising that the interaction between the physical and biological environment, and the variation within species drive the development of adaptations that allow organisms to survive in their specific habitats (Egerton 2011; Mueller 2019; Watts et al. 2019). Indeed, a major goal of evolutionary ecology has been to study how these interactions shape the physiological, morphological, behavioural and life history traits of organisms, revealing the intricate processes by which the environment moulds species over time. Environmental variation is indeed pervasive and can affect diversity across the different levels of ecological organization (e.g., Scheffers et al. 2016; Bernhardt et al. 2020). Moreover, it can also shape evolutionary

processes that generate and maintain this diversity (e.g., Hoffmann and Sgrò 2011; Bell 2013; Carlson et al. 2014; McGuigan et al. 2021).

The adaptation to variable environments is a key process in the survival of organisms, where these adjust their phenotypes in response to environmental fluctuations. While the idea that fluctuations in the immediate environment produce a shift in the direction of selection –favouring those genotypes responsible for the best-fit phenotypes– dominates the view of adaptive tuning, not all adjustments are genetic in origin. There are adaptive responses, such as phenotypic plasticity or bet-hedging strategies –among the best known (Meyers and Bull 2002; Botero et al. 2015)–, that allow genotypes to respond rapidly to environmental changes (e.g., Morgan and Watkins 2019; Ashe et al. 2021; Bautista and Crespel 2021), without depending on genetic mutations on which natural selection operates in the longer term. Among such a kind of non-genetic responses, transgenerational effects may play a crucial role in the medium term. These effects allow ancestors in a given generation to influence the phenotype of their descendants without directly altering the DNA (Chevin et al. 2010; Bonduriansky et al. 2012, 2016). Through epigenetic mechanisms, modifications in the environment, or even parental behaviour, adaptations are passed on that help future generations cope with changing conditions, demonstrating the ability of organisms to rapidly respond to their environment beyond classical genetic evolution.

Transgenerational effects vary in their conception across scientific disciplines and areas of biology due to differences in mechanisms, focus, and study approaches (Badyaev and Uller 2009; Mousseau et al. 2009). In evolutionary ecology these effects are often viewed, –through the lens of adaptation and evolutionary trade-offs– as representing an evolved commitment between selective pressures acting on ancestors and their descendants that impacts survival and reproductive success in variable environments (Mousseau and Fox 1998a, 1998b; Badyaev and Uller 2009; Mousseau et al. 2009). Among adaptive non-genetic transgenerational effects, transgenerational plasticity is a type of phenotypic plasticity that occurs across generations and exploits time correlations in the environment between successive generations. It allows ancestors in a given generation, mediated by environmental cues or the conditions they experience (Yin et al. 2019; Colicchio and Herman 2020; Dury and Wade 2020), to anticipate the environment in which their offspring will develop and influence these future generations without directly altering the DNA (Chevin et al. 2010; Bonduriansky et al. 2012, 2016). Therefore, the adaptive value of transgenerational plasticity is linked to the predictability of the environment (Uller 2008; Uller et al. 2013; Engqvist and Reinhold 2016; Proulx and Teotónio 2017). Because of the time correlation, ancestors have reliable information about the environmental conditions that their offspring are most likely to encounter, and thus have the potential to modify offspring phenotypes across generations to

improve adaptation to the environment and increase fitness (Mousseau and Dingle 1991; Agrawal et al. 1999; Uller 2008; Herman and Sultan 2011; Dantzer et al. 2013; Donelan et al. 2020). Over the past recent decades, evolutionary ecologists have broadly studied the extent to which transgenerational effects enable offspring to express adaptive plasticity in temporally heterogeneous environments (e.g., Mousseau and Fox 1998a, 1998b; Marshall and Uller 2007; Leimar and McNamara 2015; Colicchio and Herman 2020). Understanding transgenerational plasticity is considered crucial for predicting how transgenerational effects influence offspring performance, population dynamics, and species' responses to rapid environmental change (e.g., Bateson et al. 2014; Guillaume et al 2016; Donelson et al 2018; Morgan and Watkins 2019; Donelan et al. 2020; Harmon and Pfennig 2021; Clement et al. 2023). However, evidence on the prevalence and strength of these effects in natural systems is still scarce (Sultan et al. 2009; Herman and Sultan 2011; Waterman and Sultan 2021; Chapter 1).

The research conducted in this thesis addresses a transgenerational plastic effect associated with the inhibition in the response to the inducers of sexual reproduction in rotifers, a case that resembles the best documented instances of the so-called transgenerational interval timers in many insects (Chapter 1). Interval timers act as endogenous timekeepers (Rensing et al. 2001), inhibiting the expression of some life history traits over a period of time. The best documented example at the

physiological level is the transgenerational inhibition of diapause in aphids, where there is a period of no response to diapause induction in the generations following that one that has already undergone this latency stage (Matsuda 2017, 2023; Reznik and Voinovich 2021). This effect is thought to be an adaptive mechanism to prevent diapause from occurring at the wrong time (e.g., at a period when environmental conditions would normally be optimal for growth and reproduction).

Diapause is a key life history trait also in facultatively sexual rotifers (e.g., Ricci 2001; Schröder 2005; Stelzer 2005; Gilbert 2007; García-Roger et al. 2019; Chapter 1). It allows rotifer genotypes to survive during periods of adverse conditions in the water column. Diapause has a programmed duration (although it can be shorter or longer) but, once this period is fulfilled, the exit of the diapause occurs when favourable conditions are restored, and a new growing season begins. These conditions will most likely continue for some time. In this scenario of persistence of favourable conditions, some authors have tried to explain the phenomenon of inhibition of the response to sex-inducing signals in post diapause generations, which has been observed in many species (Gilbert 2002, 2003, 2017; Gilbert and Schröder 2004; Seudre et al. 2019; Chapters 3 and 5). The theoretical study by Serra et al. (2005) demonstrated the advantage of a genotype transiently insensitive to sex induction signals in populations with asynchronous diapause emergence, when other genotypes have already begun to colonize the water column and reached

high enough population densities as to induce sexual reproduction. The advantage of this strategy relies on the expectative that favourable conditions will persist for a longer time, so that rotifers can anticipate what their environment will be like in the near future. On the contrary, in unpredictable environments, the transgenerational effect would be maladaptive (Chapter 3).

The study presented in this thesis focused on the facultative sexual rotifer *Brachionus plicatilis* (Chapter 2) and provides the first empirical evidence of the adaptive value of this long-lasting transgenerational inhibitory effect on sex induction in relation to the degree of environmental predictability and explores its molecular basis (Chapters 3 and 4). The approach is essentially correlational (observational), which is a key approach to understanding the relationships between phenotypic, genotypic and ecological traits. This approach has deep historical roots in evolutionary ecology and has been used successfully in many studies of population differentiation and local adaptation (for a general perspective, see Mazer and Damuth 2001), including several in rotifers in relation to environmental unpredictability (e.g., Franch-Gras et al. 2017b, 2019; Jezkova et al. 2022). Specifically in this thesis, it was addressed how a trait with transgenerational expression, the insensitivity to sexual reproduction inducers, and a key environmental variable, the degree of predictability characteristic of the habitat, co-vary among distinct populations and among distinct genotypes within each population

(Chapter 3). While correlations do not imply causation, identifying key relationships among variables helps to test and generate new hypotheses about the ecological and evolutionary processes that shape adaptive responses. The results of this approach (Chapter 3) were consistent with the hypothesis that rotifer populations in predictable water bodies exhibit inhibition of sexual reproduction extending over several generations. Although some variability was observed among rotifer genotypes within a single population, these conformed to the expected pattern, which would allow them to exploit the growing season even if other conspecifics are already present in the water column by taking advantage of the certainty that the growing season will still persist for some time. This strategy would not be adaptive in uncertain environments, and thus less inhibition was found in the genotypes of populations inhabiting water bodies with greater unpredictability in the interannual pattern of distribution of hydroperiods. The results obtained showed that rotifer genotypes from these populations conveniently induce sexual reproduction earlier, which will most likely ensure the production of diapausing eggs before an unexpected end of the growing season.

Despite the results showing a pattern in the relationship between the effect of inhibition of sexual reproduction in the first generations after diapause and environmental predictability, some populations deviated from what was expected. This may be accounted for several factors. On

one hand, considering the point of view of the organism on focus with respect to the variation studied (see Chapter 2), it is possible that the environmental predictability metric used in this thesis is a rough approximation to that actually perceived by *B. plicatilis* in its natural habitat. In fact, including relevant measures of predictability is one of the considerations that have been proposed as key to investigations of transgenerational effects concerning environmental variation (e.g., Donelson et al. 2018). Of course, there are multiple components of unpredictability in rotifer habitats (e.g., extreme events in environmental conditions or the unexpected presence of antagonists). However, even looking only at the uncertainty associated to the hydroperiod, one might consider whether not only the interannual pattern of hydroperiod distribution (see Chapter 2) but the variation in its duration would also have an effect. This general discussion section raises the possibility of exploring other measures of habitat stability such as the variation in the length of hydroperiods. But there would be more to come, since other confounding factors could also be at work. The length of the hydroperiod, on average, could also have an effect on the number of generations in which the transgenerational effect could be observed by masking the raw impact of the degree of environmental predictability. It is worthy to note that the longer the hydroperiod, the greater the number of generations in which the response to inducers of sexual reproduction could be inhibited. The results of this thesis point in this direction (Chapter 3).

Notwithstanding, the panoply of confounding factors in the natural environment is simply overwhelming. Therefore, an alternative approach to flawlessly test the adaptive value of the transgenerational effect would be through laboratory manipulation of the predictability regimes of experimental populations. This would be an experimental evolution approach, like those that have already been done for other rotifer life history traits in relation to hydroperiod length (Smith and Snell 2012) or to environmental predictability itself (Tarazona et al. 2017). Interestingly, rotifers have a set of features that make them suitable models in experimental evolution studies (i.e., rapid adaptive response), as stressed in recent reviews (Fussmann 2011; Declerck and Papakostas 2017; Serra et al. 2019). This type of experimental approach –which would be the subject of further research– by allowing to control for potentially confounding effects would enhance research into the transgenerational plasticity on sexual reproduction in rotifers and, therefore, complement the correlational analysis developed in this thesis.

The results of the thesis also revealed that the transgenerational effect of insensitivity to the inducers of sexual reproduction does not vary with either offspring laying order from the stem female or her fecundity (Chapter 5). Note that to explore this effect, the number of offspring on which the self-conditioning bioassays in the common garden experiment were performed (Chapter 2) was expanded to the limit of the reproductive period of each stem female, although the number of

populations and generations under study had to be scaled down to make the experiment logistically manageable. Thus, a single population was chosen in which the inhibitory effect on sexual reproduction in the first generations after hatching from the diapausing egg had previously been observed (see Chapter 3). Overall, the above-mentioned results do not support the mechanism of transmission of a cytoplasmatic component as originally proposed by Gilbert (2002, 2003, 2017) to explain this non genetic transgenerational effect, and thus other alternative molecular mechanisms of epigenetic control not relying in extranuclear factors were explored.

Uncovering how plastic changes of a trait that occurring over several generations in response to environmental conditions are coordinated at the molecular level is a challenge that combines evolutionary ecology with molecular genomics. However, despite the importance of understanding these molecular processes for thoroughly assessing the capability to adapt to environmental changes, the current knowledge of the molecular underpinning of transgenerational plasticity is scarce in many cases (e.g., Herman and Sultan 2011; Donelson et al. 2018; Bell and Hellmann 2019; Bonduriansky 2021; Zetzsche and Fallet 2024). This is not surprising, considering that mechanistic molecular genetic approaches and evolutionary ecological approaches on the study of transgenerational plasticity have been carried out largely in isolation from each other. At the molecular level, a key mechanism of phenotypic plasticity is the

regulation of gene expression, where certain genes are either upregulated or downregulated. This adjustment controls regulatory pathways or directly influences the expression of traits, enabling organisms to respond effectively to shifts in their environment. A salient aspect of this thesis is that it aimed to link gene transcription (Chapter 4) and phenotypic responses (Chapter 3) regarding a relevant ecological trait –the initiation of sexual reproduction after experiencing diapause– and ascertain if these responses differed depending on the predictability of the environment. The purpose was to shed light on the mechanisms involved in the transgenerational effect and their persistence over multiple generations (Chapters 4 and 5). The conducted analyses were based on RNA reverse transcription in real-time quantitative PCR (RT-qPCR) –a technique that has proven useful in other recent studies on transgenerational effects (Im et al. 2023; Pan et al. 2023; Yin et al. 2023)– and revealed an increasing expression of a gene related to the synthesis of the sex hormone estradiol across generations in clones from rotifer populations inhabiting predictable environments, whereas no differential expression between early and late generations was observed in the clones from populations inhabiting unpredictable ponds (Chapter 4). This suggests a possible role of estradiol in the progressive disinhibition of sex across generations after diapause and points out that evolution of plasticity in response to the degree of environmental predictability can influence expression patterns of genes. However, the search for a

mechanism of epigenetic inheritance underlying the transgenerational effect by regulating gene expression was not successful. Among the main genome-associated inherited mechanisms of gene regulation (Jablonka and Raz 2009; Adrian-Kalchhauser 2020) –which include DNA methylation and histone modifications– the research in this thesis focused on a housekeeping methylation for the silencing of genes related to sexual reproduction by looking on the differential expression of a methyltransferase gene. DNA methylation is indeed a widely recognized epigenetic mechanism that plays a critical role in regulating gene expression, even in the context of transgenerational plasticity (e.g., Angers et al. 2010; Herman and Sultan 2016; Matsuda et al. 2020b; Bogan et al. 2023). Furthermore, the choice of methyltransferase DNMT2 was well substantiated by previous evidence in rotifers (Kim et al. 2016) and specifically in the model species *B. plicatilis* (Chapter 4). Nevertheless, DNMT2 gene (*meth*) expression did not vary across generations nor in relation to habitat predictability. These findings leave open the question of what inherited gene regulation mechanism underlies the transgenerational effect of sex inhibition in rotifers. A spontaneous demethylation process starting after the exit of the diapause would be plausible, as it would be also the involvement of histones (Chapter 4). Thus, an exciting field for future studies lies within investigating the inherited gene regulation mechanisms behind the transgenerational effect. Advancements in this mechanistic understanding will be a crucial

step towards deciphering the complex interplay between epigenetics and ecology in driving transgenerational adaptation to time-fluctuating environments.

Conclusions

The main conclusions derived from this thesis are enumerated below:

1. A non-genetic transgenerational inhibitory effect affected the response to sex inducing cues over several clonal generations after leaving diapause in *Brachionus plicatilis* populations.

2. The transgenerational effect differed among populations from natural habitats varying in the degree of environmental predictability regarding the interannual distribution of hydroperiods.

3. Rotifer clones from more predictable ponds tended to be unresponsive to sex inducers for more generations after leaving diapause than clones from unpredictable ponds. Therefore, the transgenerational inhibition of sexual reproduction has evolved in populations of habitats where the length of the growing season is more predictable on an interannual scale.

4. The longer the growing season, the more prolonged was the transgenerational inhibition effect; that is to say it affected a greater number of asexual generations. This suggests that the transgenerational effect will allow a fuller exploitation of the growing season, maximising the production of diapausing eggs at the end of the season.

5. There was substantial within-population clonal variation in the transgenerational inhibition of sex response. This genetic variation implies that potential for future adaptation exist in the studied populations.

6. Rotifer clones from unpredictable ponds did not exhibit transgenerational inhibition of sex induction. This could allow to produce diapausing eggs as soon as possible reducing the risk of extinction if the growing season is unexpectedly short.

7. Reverse transcription in real-time by quantitative polymerase chain reaction (RT-qPCR) allowed to track expression of genes related to synthesis of sex hormones and to a potential epigenetic signalling mechanism along successive generations from diapausing eggs in clones of *B. plicatilis*.

8. Expression level of 17-*β*-dehydrogenase 12 enzyme (*edh*) gene, related to the synthesis of estradiol, increased across generations in clones from rotifer populations inhabiting the more predictable environments. This provides a putative role of estradiol in the observed transgenerational effect.

9. The increase of *edh* gene expression across generations in populations from predictable environments was correlated with a high investment in sexual reproduction in late generations. Therefore, at the molecular level, both the timing of sex and the investment in it can be tracked by quantifying the expression of this gene.

10. Expression level of DNA methyltransferase (*dmnt2*) gene did not show differences neither across generations nor regarding the degree of environmental predictability. This does not support the conjecture about a housekeeping methylation for the silencing of genes related to sexual reproduction.

11. No increase of sexual response to sex induction in offspring was detected with either laying order or fecundity of females both stem and of a generation far away from the hatching of the diapausing egg. This does not support an underlying mechanism for the

transgenerational inhibitory effect mediated by cytoplasmatic factor being transferred from mother to offspring.

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Appendixes

Appendix 1. The study sites

The main characteristics of the eight Mediterranean saline ponds and lakes from Eastern Spain (Albacete, Castilla La Mancha) studied in this thesis are described. Ponds are ordered according to their degree of environmental predictability (Table 2.1 in this thesis; but see also Franch-Gras et al. 2017a, 2017b).

Pétrola

Pétrola (Figure A.1.1) is a steppe, temporary, shallow (0.7 m depth; Montes and Martino 1987), hypersaline (with salts such as magnesium chlorides and sulphates) and eutrophic pond (Soria et al. 1998). According to Brock's et al. (2003) classification system, it is considered a semi-permanent pond. Pétrola pond has a tectonic origin and belongs to the hydrographic basin of the Júcar River within the so-called Sector Salino de Pétrola-Corral Rubio-La Higuera (DGOH-C.H.S, 1997; López et al. 2004). The pond is the depression of an endorheic basin with a surface area of 43 km^2 (Gómez-Alday et al. 2008; Menchén et al. 2020). It is a natural habitat of high environmental value due to the community of organisms living there (Cirujano Bracamonte 1990; Soria et al. 1998; Picazo 2013;

Verde et al. 2017), e.g., birds like the western marsh harrier (*Circus aeruginosus*). Pétrola was declared as a "Site of Community Importance" at EU level in 1997, "Natural Reserve" by Junta de Castilla-La Mancha Regional Government (DOCM 26/2003).

Figure A.1.1. The image shows an aerial photograph of Pétrola pond (Pétrola, Albacete) from Google Maps (38º50'16.82"N, 1º33'49.22"W) as the main view, complemented by a terrestrial photograph inserted in the left-lower corner. *Source: Evolutionary Ecology Laboratory, University of Valencia.*

Salobralejo

Salobralejo is an endorheic, shallow, hypersaline, and eutrophic pond (Cirujano Bracamonte 1990; Milán et al. 2001; Picazo 2013). Its soil is

sandy-clayey, having been traditionally used for cereal cultivation (López et al. 2015). It has slightly turbid waters (Reed 1998) and its hydrological regime has been characterized as semi-permanent (García-Roger et al. 2006). Apart from the rotifer species under study in this thesis, the following rotifers have also been described in this pond: *Brachionus quadridentatus* and *Testudinella patina.* Copepods such as *Acanthocyclops robustus* and *Arctodiaptomus salinus*, and cladocerans such as *Daphnia longispina* and *Phrixura rostrata* are part of the zooplankton community in its waters. (Lapesa 2004).

Figure A.1.2. The image shows an aerial photograph of Salobralejo pond (Higueruela, Albacete) from Google Maps (38º54'52.11"N, 1º28'6.95"W) as the main view, complemented by a terrestrial photograph inserted in the left-lower corner. *Source: Evolutionary Ecology Laboratory, University of Valencia*.

Atalaya de los Ojicos

Atalaya de los Ojicos is a shallow and hypersaline pond that belongs to the Corral Rubio-La Higuera endorheic complex formed by a total of 18 depressions and ponds with different ecological characteristics (Cirujano Bracamonte 1990). It is located within the hydrographic basin of the Segura River at an altitude of 880 m (López et al. 2004). Atalaya de los Ojicos is characterized by abundant vegetation on its bottom (e.g., *Lamprothamnium papulosum*) and margins (e.g., *Scirpus marilimus*), when plenty of water (Cirujano Bracamonte 1990).

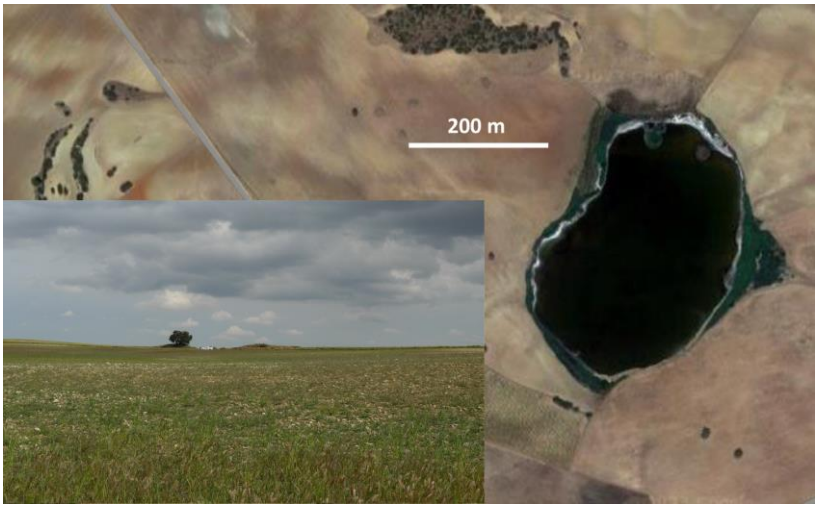

Figure A.1.3. The image shows an aerial photograph of Atalaya de los Ojicos pond (Corral Rubio, Albacete) from Google Maps (38º46'20.97"N, 1º25'49.12"W) as the main view, complemented by a terrestrial photograph inserted in the left-lower corner. *Source: Evolutionary Ecology Laboratory, University of Valencia*.

Hoya Turnera

Hoya Turnera also belongs to the Corral Rubio-La Higuera endorheic complex. It is very small pond, and it usually has a high density of emergent macrophytes. Its salinity ranges from 1.84-3.06 g/L (see Table 4.1).

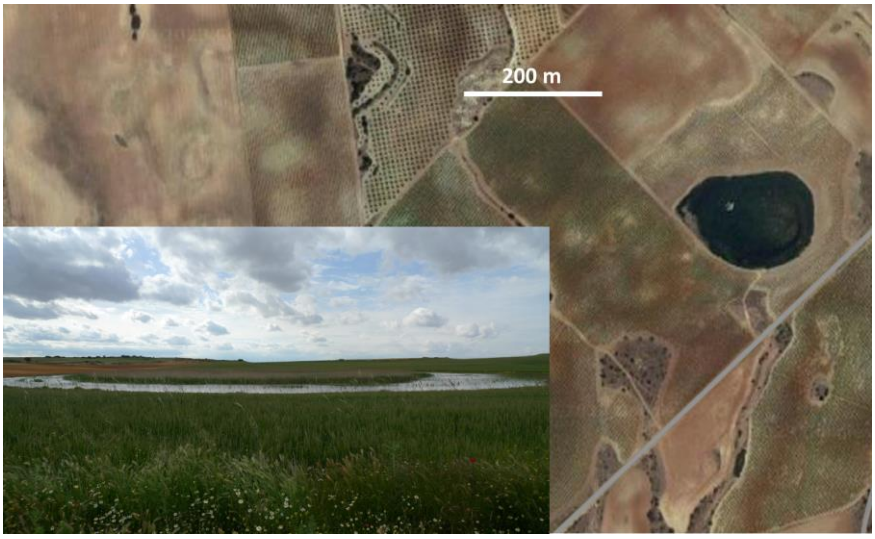

Figure A.1.4. The image shows an aerial photograph of Hoya Turnera pond (Albacete) from Google Maps (38º46'31.19"N, 1º24'37.41"W) as the main view, complemented by a terrestrial photograph inserted in the left-lower corner. *Source: Evolutionary Ecology Laboratory, University of Valencia.*

Hoya Yerba (a.k.a. Hoya La Hierba, Hoya de la Yerba)

Hoya Yerba also belongs to the Corral Rubio-La Higuera endorheic complex and is located at an altitude of 840 m (López et al. 2004). Its salinity ranges from 2.84-16.5 g/L (see Table 4.1). The pond supports an important diversity of avifauna.

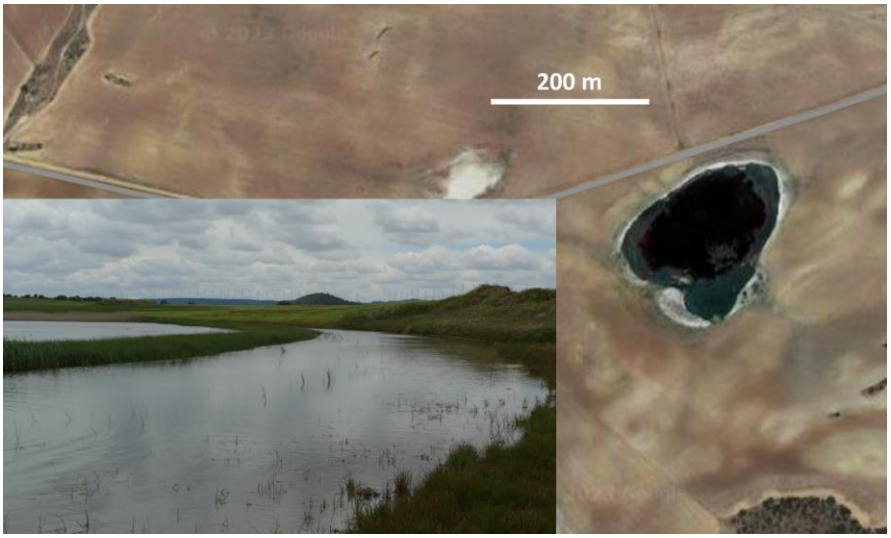

Figure A.1.5. The image shows an aerial photograph of Hoya Yerba pond (Albacete) from Google Maps (38º46'46.02"N, 1º26'6.60"W) as the main view, complemented by a terrestrial photograph inserted in the left-lower corner. *Source: Evolutionary Ecology Laboratory, University of Valencia*.

Hoya del Monte

Hoya del Monte belongs to the Corral Rubio-La Higuera endorheic complex and is located at an altitude of 900 m (López et al. 2004). It is a subsaline depression, strongly nitrified by grazing (Cirujano Bracamonte 1990). It shows abundant marginal vegetation dominated by *Juncus bufonius* (Cirujano Bracamonte 1990).

Figure A.1.6. The image shows an aerial photograph of Hoya del Monte pond (Corral Rubio, Albacete) from Google Maps (38º50'44.87"N, 1º26'38.70"W) as the main view, complemented by a terrestrial photograph inserted in the left-lower corner. *Source: Evolutionary Ecology Laboratory, University of Valencia*.

Hoya Chica (a.k.a. Laguna Chica de Recreo)

Hoya Chica belongs to the Corral Rubio-La Higuera endorheic complex, located at an altitude of 860 m within the hydrographic basin of the Segura River (Cirujano Bracamonte 1990; López et al. 2004). It is a depression of subsaline nature with shallow waters (2-8 cm). In the pond there is abundant aquatic vegetation such as, *Tolypella hispanica, Scirpus maritimus* or *Polygonum marinum* (Cirujano Bracamonte 1990).

Figure A.1.7. The image shows an aerial photograph of Hoya Chica pond (Corral Rubio, Albacete) from Google Maps (38º49'46.22"N, 1º27'49.74"W) as the main view, complemented by a terrestrial photograph inserted in the left-lower corner. *Source: Evolutionary Ecology Laboratory, University of Valencia*.

La Campana (a.k.a. Lagunas del Recreo)

La Campana belongs to the Corral Rubio-La Higuera endorheic complex and is located at an altitude of 940 m within the hydrographic basin of the Segura River (López et al. 2004). This pond is also called “Lagunas” because of the two sub-basins that are part of the pond.

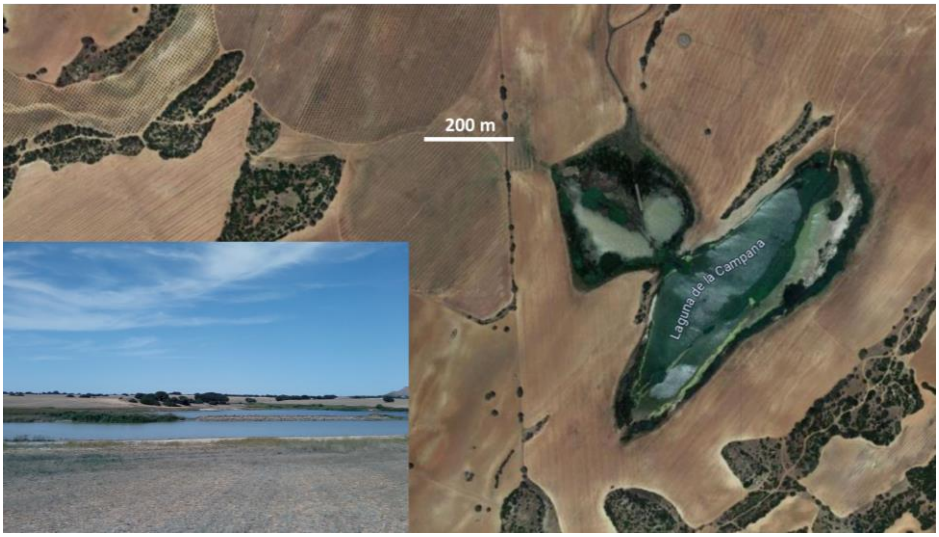

Figure A.1.8. The image shows an aerial photograph of La Campana pond (Albacete) from Google Maps (38º51'29.06"N, 1º29'36.97"W) as the main view, complemented by a terrestrial photograph inserted in the left-lower corner. *Source: Evolutionary Ecology Laboratory, University of Valencia.*

Appendix 2. Anatomy of the rotifer *Brachionus plicatilis*

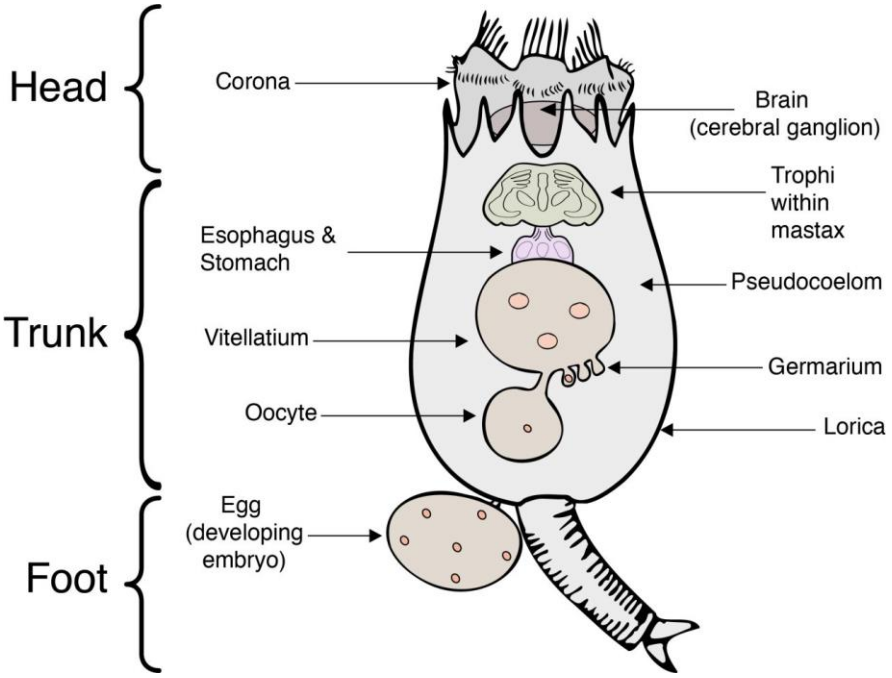

Figure A.2. Simplified illustration of the anatomy of an adult female of rotifer *B. plicatilis* in a ventral view carrying one egg.

Appendix 3. Continuous culture system: chemostat

As pointed out in Chapter 2, for the accurate control of food quality in experiments, the microalgae used in the rotifer culture medium in the experiments of propagation of generations were raised in a continuous flow culture in a chemostat (Figure A.3) with a dilution rate of 0.295 per day. The chemostat consisted in a 2-L bioreactor for the growth *T. suecica*., a peristaltic pump and a reservoir). In the reactor, fresh medium was continuously added at a defined rate, matching the rate at which culture was removed. This ensured that the volume remained constant, adhering to the fundamental principles of such systems (Gresham and Dunham 2014) under standard culture conditions (see Chapter 2). In this way, a steady state is achieved, whereby the rate at which the population of microalgae grows is equal to the rate at which the culture is diluted (0.295 per day in this case). These rates enable the growth of the microalgae to grow exponentially under constant biological and physicochemical conditions (Pir et al. 2012). The microalgae were grown in the reactor at high cell concentration (around 800,000 – 1,000,000 cells m/L). The fresh medium in the reservoir was composed of 12 g/L, artificial seawater plus nutrients (see standard conditions Chapter 2) and was provided to the reactor at a 0.410 mL/min rate using the peristaltic pump. The microalgae, to be used as rotifer culture medium in the experiments, were removed from the reactor at the same

constant flow rate, maintaining the volume constant. All components of the chemostat were connected through silicone tubes (0.89 mm in diameter). Air pumps were used to aerate and maintain culture homogeneity, both in the reactor and the reservoir. To avoid contamination, a 0.2-µm filter (PTFE-Filter; Sartorius Midisart) was used to sterilise air before entering in the system. The chemostat was held in a walk-in growth chamber at standard conditions. The concentration of microalgae in the effluent was calculated using an automatic cell counter (Celeromics Technologies S.L.) and then adjusted by dilution with 12 g/L salinity water until it reached concentrations of 250,000 or 500,000 cells m/L to be used as food for *B. plicatilis* in the bioassays.

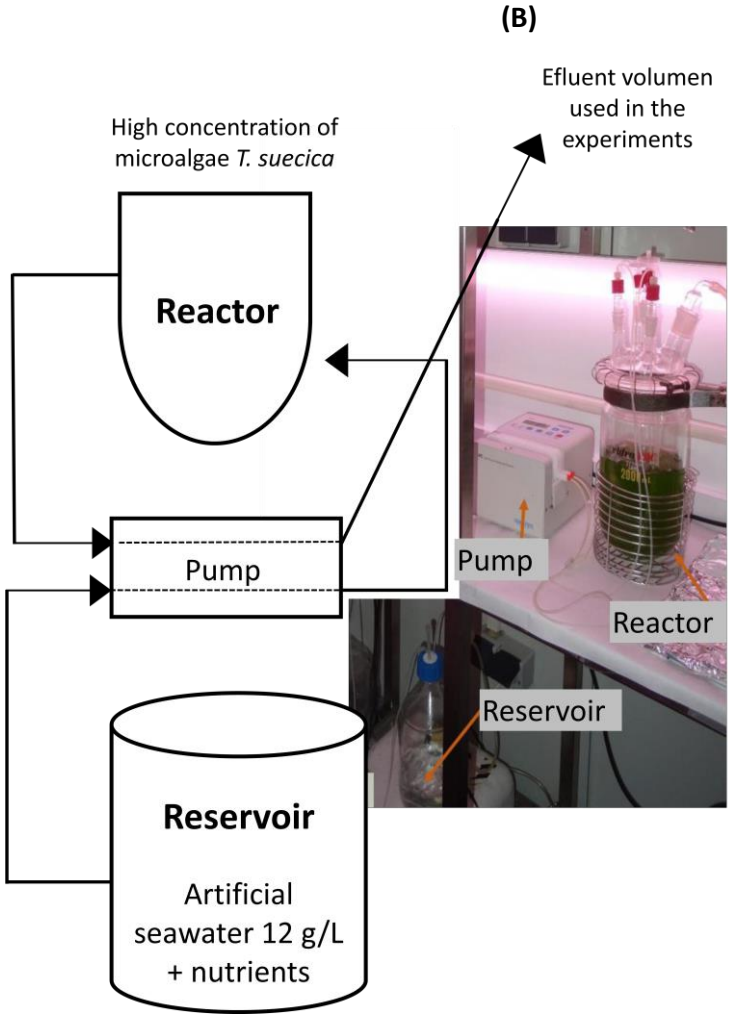

Figure A.3. Chemostat system **(A)** drawing and **(B)** photographs. From the reservoir with artificial seawater plus nutrients, fresh medium is supplied by a pump to the reactor where the algae are growing at high concentration. Via the pump comes out the volume of microalgae (effluent volume) at a convenient concentration as the convenient input to be used in the experiments. The silicone tubes connecting the reservoir, the reactor and the pump are represented by arrows (modified from Tarazona 2018).

Appendix 4. Supplementary material to Chapter 3

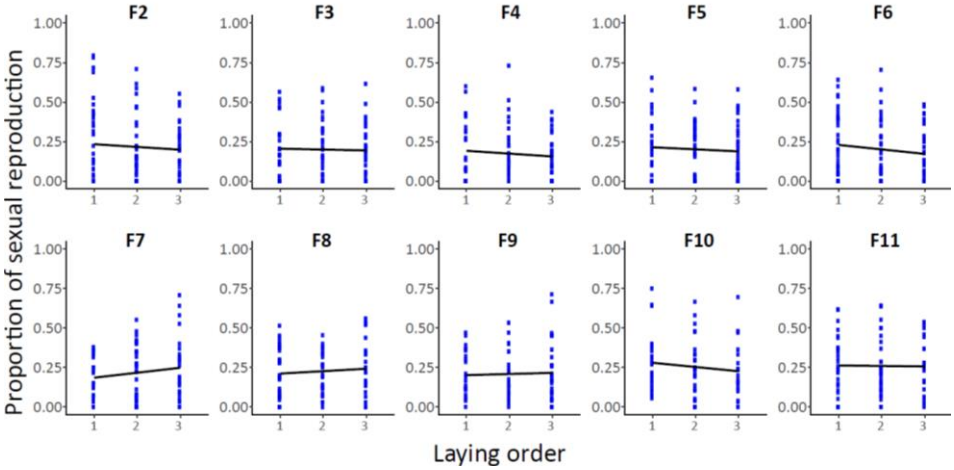

Figure A.4. Effect of laying order on the proportion of sexual reproduction (PSR) in each generation (F2-F11) for the clones of the eight studied natural populations of rotifer *B. plicatilis*. Black lines in each plot represent the linear regression fit. In all cases, the correlation (unidirectional, one-tailed test) was not significant after Bonferroni's correction ($p > 0.005$).

Table A.4. Results of correlation analyses between the proportion of sexual reproduction (PSR) and two groups of generations for the eight studied populations of the rotifer *B. plicatilis*. The *early* group averaged F2 and F3 and the *late* group averaged F10 and F11. The significance of Pearson's correlation coefficients (r) was assessed using a Bonferroni-corrected p value of 0.005. The relationship between the PSR and culture density was fitted by means of a linear model to obtain the intercept (a), slope (b), and their respective 95% confidence intervals. Ponds were ordered according to their level of predictability (Franch-Gras et al. 2017a).

Pond	Group	*a*	95% CI[*a*]		*b*	95% CI[*b*]		*r*	*p*
			LL	UL		LL	UL		
Pétrola	Early	0.3027	0.0917	0.5137	-0.0223	-0.0450	-0.0001	-0.3746	0.9752
Pétrola	Late	0.1005	-0.1280	0.3289	0.0015	-0.0253	0.0282	0.0263	0.4550
Salobralejo	Early	0.1018	-0.0876	0.2912	0.0018	-0.0155	0.0192	0.0404	0.4146
Salobralejo	Late	0.1953	-0.0277	0.4184	0.0055	-0.0179	0.0290	0.0930	0.3157
Atalaya de los Ojicos	Early	-0.0273	-0.1064	0.0517	0.0030	-0.0039	0.0099	0.3638	0.1679
Atalaya de los Ojicos	Late	0.3661	0.0431	0.6890	-0.0234	-0.0544	0.0076	-0.5591	0.9412
Hoya Turnera	Early	-0.0299	-0.1967	0.1368	0.0098	-0.0057	0.0254	0.2475	0.1021
Hoya Turnera	Late	0.3289	0.1171	0.5407	-0.0155	-0.0355	0.0045	-0.3324	0.9394
Hoya Yerba	Early	0.2022	-0.1455	0.5498	0.0184	-0.0156	0.0524	0.2326	0.1370
Hoya Yerba	Late	0.2630	-0.4488	0.9749	0.0020	-0.0643	0.0683	0.0299	0.4720
Hoya del Monte	Early	0.3521	0.0733	0.6310	0.0012	-0.0247	0.0272	0.0210	0.4611
Hoya del Monte	Late	0.1620	-0.0867	0.4107	0.0249	-0.0020	0.0517	0.4541	0.0335
Hoya Chica	Early	0.1971	-0.1354	0.5296	0.0046	-0.0253	0.0345	0.0681	0.3760
Hoya Chica	Late	0.1109	-0.0387	0.2605	0.0109	-0.0056	0.0273	0.2866	0.0925
La Campana	Early	0.1449	-0.2108	0.5006	0.0217	-0.0164	0.0597	0.2715	0.1235
La Campana	Late	0.3133	0.0611	0.5656	0.0204	-0.0074	0.0482	0.3160	0.0709

Appendix 5. Supplementary material to Chapter 5

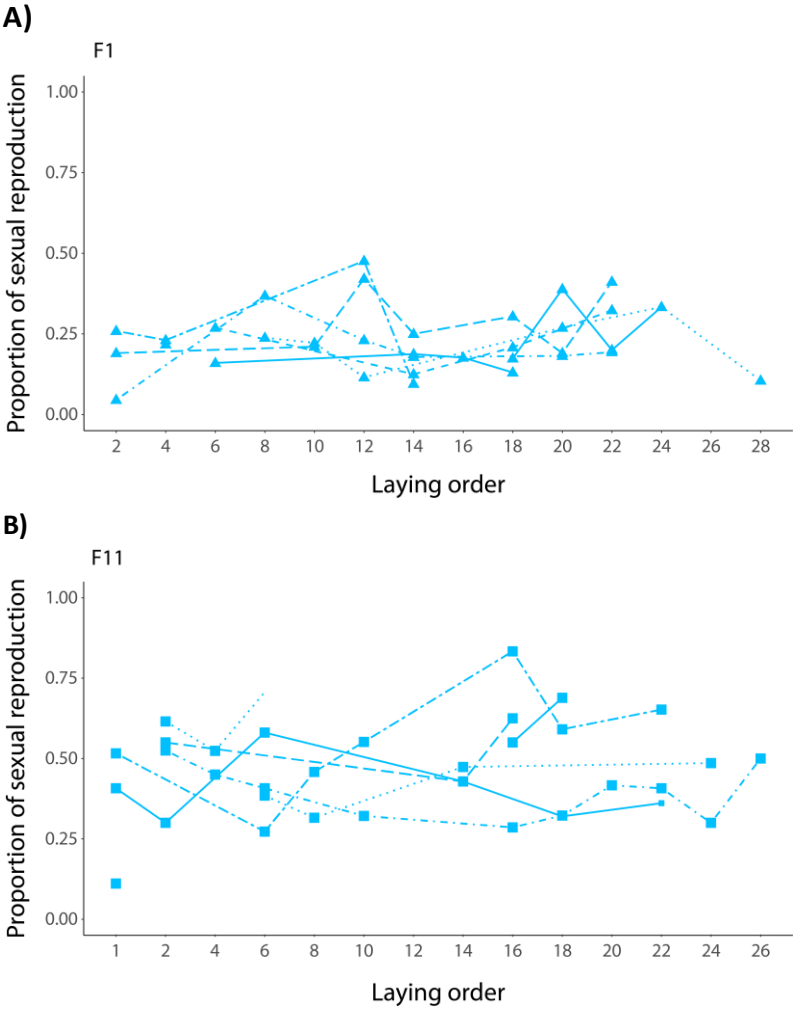

Figure A.5. Variation in the proportion of sexual reproduction (PSR) across laying order for each of the eight clones studied from Hoya Turnera (HTU) *B. plicatilis* population. **A)** First generation (F1), corresponding to offspring from the stem female, and **B)** Eleventh (F11), corresponding to daughters of F10 generation females.