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Adaptation to environmental unpredictability in rotifers: an experimental evolution approach

Eva del Pilar Tarazona Castelblanque

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Directors:

María José Carmona Navarro

Eduardo Moisés García Roger

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Signat: Eva del Pilar Tarazona Castelblanque

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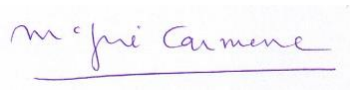

María José Carmona Navarro
Catedràtica d'ecologia
Universitat de València

Eduardo Moisés García Roger
Professor contractat doctor
Universitat de València

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Adaptation to environmental unpredictability in rotifers: an experimental evolution approach

I call this experiment "replaying life's tape". You press the rewind button and (…) go back to any time and place in the past (...). Then let the tape run again and see if the repetition looks at all like the original

Stephen Jay Gould

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Summary in English

Introduction

Organisms inhabit environments in which abiotic and biotic conditions are not constant, but vary temporally and/or spatially. Such environmental fluctuations are ubiquitous in nature and impose strong selection pressures to which organisms must adaptively respond. Consequently, unveiling the adaptive response of organisms to cope with heterogeneous environments has become a central topic in fundamental and applied evolutionary ecology.

Dispersal allows organisms to “travel” in space and/or time in order to escape from unsuitable conditions, and it is dependent on the temporal and spatial heterogeneity of the environments. This has been associated in some organisms with the production of resistant, dormant stages that can remain in dormancy for a variable periods of time. These dormant forms represent a major and widespread adaptation that occurs in the life histories of many organisms in order to prevent population extinction and to promote dispersal and colonization of new environments. Diapause is a type of dormancy, which is characterized by a temporal suspension of the metabolic activity, arrested development, and an increased resistance to stress. Depending on species and habitats, diapause can occur at different stages of the life cycle (e.g., eggs,

larvae, pupae or adults, for instance in copepods and insects). The entrance into diapause –and their duration– depends on an endogenous control, and it remains –even if suitable conditions resume– until specific cues disrupts it. Given that many diapausing stages do not hatch when suitable environmental conditions return, and remain viable in the sediment, they contribute to the formation of the so-called “egg banks”. These diapausing egg banks act as reservoirs of genetic diversity, promoting the persistence of species and the maintenance of the phenotypic and genetic variability within populations. Thus, diapause has been considered as a strategy to avoid risk through time, by working as a sort of “time machine”.

This thesis focuses on temporally varying environments, which are characterized by the alternation between suitable and unsuitable environmental conditions. Such alternation can be considered as predictable, if it occurs recurrently; or unpredictable, if the alternation follows a random pattern. At this respect, diapause can be the subject of different adaptive responses by organisms inhabiting temporally varying environments. Organisms can cope with environmental variation through phenotypic plasticity –i.e., a single genotype producing different phenotypes depending on particular environmental conditions– which is expected to occur when environments fluctuate predictably, and changes do not occur too much rapidly. When environmental fluctuations occur

slowly, a second type of expected response is adaptive tracking, in which natural selection acting recurrently on the standing heritable variation among individuals leads to genetic evolution. Finally, rapid and unpredictable environmental change is expected to favor bet hedging, a risk-spreading life-history adaptation. Bet hedging reduces temporal variation in fitness under conditions of unpredictable environmental variance. It occurs when a selected trait in a genotype reduces fitness variance at the cost of a decrease in the arithmetic mean fitness, but maximizing geometric mean fitness (i.e., the measure for long-term fitness). Bet hedging has been mostly considered as a maternal strategy in which two main modes have been described. A first mode, called *conservative bet hedging*, takes place when a single genotype produces a unique low-risk phenotype in its whole offspring to reduce temporal variance in reproductive success. A second mode, *diversified bet hedging,* occurs when a single genotype produces diverse phenotypes among its offspring in advance of future unpredictable conditions. Both types of bet hedging are apparently suboptimal under the averaged environmental conditions, but increase the long-term population growth rate. Despite bet hedging has been well developed theoretically in evolutionary ecology, the empirical evidence is still scarce.

In natural populations, environmental unpredictability can act on several life-history traits of an organism, especially in those with

complex life histories. Diapause is a classic example of a major life history component in which different traits can help to cope with environmental unpredictability. One of these traits is the timing of entering diapause. In temperate zones, the timing of switching from active to diapausing stages is a critical component of fitness. The production of diapausing stages hould to occur before the beginning of unsuitable conditions, since these stages are essential for the future survival of a genotype. The assignation of resources to produce diapausing stages involves costs to the organisms since it implies a lower inversion on current population growth. However, if suitable conditions remain, an early investment in diapause can be sub-optimal, since the potential for further population growth is reduced. Similar trade-offs also occur with respect to another diapause related trait: the timing of exiting from diapause. Under suitable conditions, exit from diapause favors the exploitation of environmental resources and enhances the competitive abilities of the reactivated organisms. However, leaving diapause can be risky if the habitat becomes unexpectedly unsuitable, and genotypes have no time enough to produce a new cohort of diapausing stages.

Experimental evolution can be a useful tool to study the adaptive responses to temporally varying environments. At the end of the twentieth century, Stephen Jay Gould in his book *Wonderful life* (1989) popularized the idea of a hypothetical experiment called “replaying life's tape”. This thought experiment involves rewinding

somehow the history of life back to its initial starting point and wonder how the story would again unfurl. This idea has been associated to experimental evolution studies as a way of testing the reproducibility of the evolutionary outcomes. Experimental evolution approach consist on the study of evolutionary processes occurring in laboratory and field populations in response to conditions imposed by the experimenter. This approach has been recognized as a powerful tool in ecology and evolution to (1) detect short-term evolutionary responses, and (2) explore the genomic basis of adaptation. The advantages of experimental evolution for tracking evolutionary trajectories are that laboratory populations evolve under controlled conditions (i.e., with defined selective pressures), and several replicates can be performed in each of the given selective regimes. These type of studies typically compare groups of populations that are derived from the same ancestral genotype and are exposed to different selective regimes through the time. The ancestral genotypes, populations or individuals used to establish the experimental populations, are key to understand the evolutionary processes that might occur throughout the experimental evolution assay. If the founding population is genetically uniform, adaptation to selective regimes could happen through the accumulation of beneficial mutations. In contrast, if the founding population is polymorphic, selection is expected to act on heritable standing variation. This type of selection is expected to

produce rapid evolution in novel environments. Therefore, laboratory experimental evolution has the potential to show adaptation to several selective pressures occurring over a relatively reduced number of generations, and has been widely applied to a phylogenetically-broad range of organisms. Among metazoans, there are several studies that have successfully applied this approach to address adaptation to environmental fluctuations. Interestingly, monogonont rotifers have been recently recognized as suitable organisms for testing evolutionary hypotheses, and have been successfully used under a variety of biotic and abiotic factors. Several features make them an excellent model for experimental evolution studies, such as (1) their small size (usually < 1 mm), which enables the establishment of large populations sizes, (2) their short generation time, and (3) the fact that many species are easily cultured in the laboratory. Rotifers are, so far, the only metazoans capable of reaching steady state population growth in continuous cultures. In this type of culture, fresh medium is renewed continuously at a constant rate, and organisms can be grown in a physiological steady state under constant environmental conditions. In addition to the above mentioned features, rotifers have (4) a complex life cycle which combines sexual and asexual reproduction. Asexual reproduction permits to easily establish clonal lineages, and thus to use genotype replicates in experiments. Instead, sexual reproduction results in the production of new

genetic variants in experimental populations, which allows selection to act on. Finally, in rotifers (5) an overlap between the evolutionary and ecological time scales has been described, what shows their potential for studying eco-evolutionary feedbacks.

The monogonont rotifer *Brachionus plicatilis* is a common inhabitant of continental, saline and brackish waters. These habitats are often characterized by strong season-to-season fluctuations in the length of the so-called growing season (i.e., the period of time in which rotifer populations are active in the water column). Therefore, the habitat can become unsuitable in an unpredictable way. These fluctuation patterns are typical in the water bodies of the Mediterranean region, which show a wide range of environmental predictability. *B. plicatilis* has a type of reproduction called cyclical parthenogenesis, in which proliferation by ameiotic parthenogenesis (asexual phase) combines with occasional bouts of male production and sexual reproduction (sexual phase). Active populations in the water column are mainly comprised of asexual females that parthenogenetically produce ameiotic subitaneous eggs. These eggs hatch into daughters that are genetically identical to their mothers. This is a kind of clonal propagation that enables fast population growth and has the potential for the colonization of new habitats by one or a few founding females. After this initial period of clonal propagation –during which population is composed by clonal lineages– sexual

reproduction is induced by a chemical signal produced by the rotifers themselves. This chemical accumulates in the medium as population density increases. Once a population density threshold is reached, asexual females are triggered to produce sexual daughters as some fraction of their offspring, so that sexual and asexual reproduction overlap. Sexual females produce meiotic haploid eggs that develop either into males or, if fertilized, into diapausing eggs. These eggs are resistant, dormant embryos, which sink and accumulate in the sediment where remain dormant for a period of variable length. Under suitable conditions, diapausing eggs are induced to hatch into asexual females that recolonize the water column, starting a new growing season. However, not all diapausing eggs hatch in the season following their production. The unhatched eggs often show prolonged diapause and accumulate forming the so-called egg banks in the sediment, waiting for suitable conditions to hatch in what seems to be a risk-spreading strategy. Diapausing egg banks are also reservoirs of the past genetic diversity and of the adaptive phenotypic variability in the population. They are essential for the long-term persistence of rotifer populations. As a whole, the monogonont rotifer life cycle is considered an adaptation to temporally varying environments.

Unravelling the molecular mechanisms and the genomic basis underlying the adaptation of organisms to their environments are key issues in evolutionary biology. In last years, the molecular and

technological advances together with the decreasing costs in high-throughput next-generation sequencing (NGS) have revolutionized the so-called “omic era”. This new scientific context has allowed to analyze large amount of data in either model or non-model species. Nevertheless, the genomic resources in non-model organisms are still scarce, what limits the ability to uncover functionally relevant genes. One of the most used “omic” technologies is genomics, which focuses on understanding the structure, function, and evolution of genomes. Through the characterization of the genomic variation, genomic technologies allow to evaluate population structure and to identify genotypes that can be responsible of adaptations. Another widely used “omic” technology is transcriptomics, which is based on the study of genes that are actively expressed at a given moment or in association to a condition (or a set of conditions) of interest. Both “omic” techniques are able to identify candidate genes that underlie the adaptations in life-history traits and therefore promote the integration between genetic and ecological data.

Objectives

This thesis takes advantage of the above-mentioned technical advances and adopts the theoretical framework of evolutionary ecology to address the adaptive responses to unpredictable environments of rotifer populations using an experimental evolution approach. The main goals of this thesis are: (1) To study

the adaptive response of life-history traits related to diapause in populations of the rotifer *B. plicatilis* under two fluctuating selective regimes (predictable vs unpredictable) simulated in the laboratory, (2) to elucidate the genomic basis of adaptation to environmental unpredictability of *B. plicatilis* populations evolved under divergent regimes, using genomic technologies, and (3) to explore the genetic expression in diapausing eggs produced by rotifer populations evolved under those two selective regimes, and subjected to two different diapause conditions (i.e., with and without an obligate period of diapause).

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 provide an overview of the general methodology used in the development of the thesis objectives. Firstly, the main biological features of the model species *B. plicatilis* were described. Secondly, experimental evolution approaches were introduced and an overview about their usefulness in ecology and evolution studies was provided. Thirdly, the experimental evolution design used throughout this thesis was explained, with details on the establishment of the laboratory populations of *B. plicatilis* which were subjected to two fluctuating environmental regimes (predictable vs unpredictable) through eight cycles of selection

interrupted by periods of habitat unsuitability. Briefly, in this chapter three laboratory populations (working as replicates) were randomly assigned to the predictable selective regime, characterized by growing seasons (i.e., hydroperiod) of constant length (28 days). The other three laboratory populations were assigned to the unpredictable regime which was characterized by growing seasons whose length varied randomly, but averaged 28 days. It can be assumed that these regimes are predictable and unpredictable, respectively, by the rotifers, so that this design mimicked selection imposed by habitat unpredictability in natural rotifer populations. Finally, in this chapter, the main molecular techniques used throughout the thesis were described (genotyping by sequencing, hereafter GBS; and RNA sequencing, hereafter RNA-Seq). GBS is a sequencing approach that relies on restriction enzymes to reduce the genome complexity and allows the identification and genotyping of thousands of single nucleotide polymorphisms (SNPs) in individuals from different populations. RNA-seq is based on the sequencing of RNA transcripts that are produced by the genome, under specific circumstances. This technique allows studying differences in gene expression patterns between populations and/or individuals within populations with different phenotypes or under different environmental conditions. This chapter ends with a description on how these techniques were performed and includes a glossary of terms.

In **Chapter 3**, the adaptation to unpredictable environments through the experimental evolution approach was studied using the rotifer *B. plicatilis* as model organism. The aim of this chapter was to test how unpredictable environments may select for traits related to diapause in populations of the rotifer *B. plicatilis*. Two life-history traits related to diapause were studied in populations evolved under two contrasting selective regimes (i.e., predictable vs unpredictable) during the course of the evolution experiment. In unpredictable habitats, the survival of rotifer populations depends on the adjustments of their life-history traits to the season-to-season variation in the length of the growing season. Given that diapause is the only way to survive unsuitable conditions between growing seasons, at least two key diapause-related traits in the life cycle of these rotifers can be considered: (1) the timing of sex (a proxy of the timing of diapausing egg production), and (2) the diapausing egg hatching fraction in a growing season (inversely related to diapause duration). Theoretical studies have proposed the timing of sex as an instance of conservative bet hedging in rotifer populations inhabiting unpredictable environments. If the end of the growing season cannot be predicted, then shifting to sexual reproduction as soon as possible can avoid the risk of unexpectedly short growing seasons (i.e., leading to a complete reproductive failure of a rotifer genotype). However, this response would be sub-optimal because a rotifer genotype producing

diapausing eggs too early, while favorable conditions still prevail, would incur a cost (i.e., the investment in diapause results in a direct reduction of the current population growth rate). In contrast, diapausing egg hatching fraction has been proposed in several theoretical studies as a form of diversified bet hedging (i.e., diapausing eggs showing different diapause durations are derived from the same genotype). According to theory, in predictable habitats, a large fraction of diapausing eggs is expected to hatch in the season following their production. Thus, hatchlings would be able to produce a new cohort of diapausing eggs during the growing season, and egg banks would be continuously renewed season after season. Instead, in unpredictable habitats, low –or even zero– recruitment to the egg bank at the end of the growing season would occur if all diapausing eggs derived from a genotype hatched from the egg bank simultaneously in an unexpectedly short growing season. Therefore, if only a fraction of the diapausing eggs hatches, a rotifer genotype would ensure its presence in subsequent favorable periods and thereby avoid extinction. During the evolution experiment, two life-history traits in each selective regime were characterized. The timing of sex was studied conducting 720 bioassays (4 cycles × 6 populations × 10 clones × 3 replicates). The evolutionary response in the diapausing egg hatching fraction was studied by performing more than 20,000 bioassays (4 cycles × 96 diapausing eggs × 10 clones × 6 population).

Both traits were estimated over the same set of clones. Rotifer laboratory populations showed rapid adaptation to unpredictable environments, displaying a divergent response in these two life-history traits. Populations subjected to the unpredictable selective regime showed both lower hatching fractions of diapausing eggs and earlier sex initiation, suggesting that bet-hedging strategies underlie adaptation to environmental unpredictability in these organisms.

In **Chapter 4,** the genomic basis of adaptation to environmental unpredictability were unravelled in the same experimental laboratory populations used in Chapter 3. By means of GBS, genome-wide polymorphisms were identified and genotyped in 169 clones from both selective regimes after the seventh cycle of the evolution experiment. These data were compared with published GBS data from 270 field clones –those used to found the laboratory populations– with the help of a draft genome assembly. As a result of GBS data analysis, 6,107 high-quality SNPs were discovered and genotyped. These SNPs were used to identify outlier loci as candidates for selection under each selective regime. There are several methods that aim to detect loci under selection. Among them, the F_{ST}-based methods compare allele frequencies among populations to identify those SNPs that have higher differentiation values than those expected under a neutral evolution model. More recently, other group of methods such as the GWAS (genome-wide

association studies) have been developed to detect signatures of selection. The GWAS methods are based on the association of phenotypic variations with their corresponding genotypes at the individual level. After applying these analyses, 76 SNPs putatively under divergent selection were identified. Three of these SNPs strongly shifted their allele frequencies in response to environmental unpredictability being candidates to be under selection. The remaining SNPs experienced parallel allele changes in experimental populations, suggesting adaptation to environmental laboratory conditions. Additionally, a genotype-phenotype association analysis revealed five SNPs associated with the two life-history traits related to diapause. Four out of these five SNPs (located in two different genes) were found to be associated with diapausing egg hatching fraction, and the other SNP left was associated to the timing of sex. In general, laboratory populations subjected to both selective regimes showed lower genome-wide pairwise F_{ST} values than when compared to the original field population. Moreover, genetic diversity was kept throughout the experiment in all laboratory populations except for one population from the unpredictable regime in which a slight decrease in genetic diversity was observed. Nevertheless, (1) the negligible loss of genetic diversity, (2) the low values of inbreeding coefficient and (3) the little change in F_{ST} between laboratory populations, suggest

that genetic drift processes were not important in comparison to selection.

In **Chapter 5**, a transcriptome analysis on the experimental rotifer populations evolved under the different selective regimes was conducted to study the molecular mechanisms associated to diapause in response to environmental unpredictability. The main objective of this chapter was to identify and quantify the expression of genes involved in both (1) the maintenance of diapause, and (2) the exit from it in *B. plicatilis*. To do that, hatching experiments and transcriptomics on diapausing eggs –produced at the end of the last cycle of selection by each of the six populations evolving under the two divergent regimes of environmental predictability– were combined. Therefore, 10,000 diapausing eggs were collected from each laboratory population and were divided into two groups to be indeed subjected to conditions either promoting or blocking hatching. Gene expression was analyzed by means of RNA-seq. After applying a set of bioinformatics analyses, a total of 3,068 differentially expressed genes were identified in rotifer diapausing eggs. The analyses performed in this chapter revealed that genes related with diapause maintenance and termination are differentially expressed in the diapausing eggs that were produced in the unpredictable regime with respect to those that were produced in the predictable one. This study extends the knowledge of the complex molecular and cellular events that take place during

diapause. Some of the genes identified here are well known in other anhydrobiotic organisms and resistance forms, but several of them are new and should be further investigated to determine their role in desiccation and stress tolerance.

Finally, **Chapter 6** provides a general discussion of the main results obtained in this thesis, proposes prospective research, and sets out the most important conclusions.

Conclusions

The main conclusions derived from this thesis are enumerated below:

1. Populations of the rotifer *Brachionus plicatilis* rapidly evolved adaptive responses to environmental unpredictability under experimental evolution.
2. Diapause-related traits –the timing of sex and the hatching fraction of diapausing eggs– evolved divergently in laboratory populations subjected to two contrasting selective regimes of environmental fluctuation (predictable vs. unpredictable).
3. The timing of sex was earlier in laboratory populations subjected to the unpredictable regime. This suggests a conservative, bet-hedging strategy that provides protection against unexpectedly short growing seasons.

4. Rotifer populations under the unpredictable regime evolved longer diapause (i.e., lower hatching fractions) than the populations under the predictable regime. This suggests a diversified bet-hedging strategy, promoting longer diapause periods and favoring the survival of rotifer populations by its persistence in diapausing egg banks when the environment is unpredictable.

5. Asynchronous diapausing egg hatching was found in populations evolved under the predictable regime. This could be interpreted as a within-season bet hedging for this trait. Such within-season, risk-spreading strategy could evolve if the occurrence of successful growing seasons is predictable but there is some uncertainty with respect to their start.

6. Genotyping by sequencing (GBS) and subsequent bioinformatics analyses provided a large number (6,107) of high quality single nucleotide polymorphisms (SNPs).

7. Three SNPs –located in three different genes– showed a higher genetic differentiation between the selective regimes than expected by chance. Therefore, they are candidates to be under selection in unpredictable environments.

8. Genotype and phenotype analyses revealed four SNPs putatively associated to diapausing egg hatching fraction, and one SNP putatively associated to the timing of sex.

9. Parallel changes in allele frequencies of a number of candidate SNPs under selection were found in the six laboratory populations, independently of their selective regime. This suggests a strong signal of adaptation to laboratory conditions during the selection experiment.

10. The negligible loss of genetic diversity, low values of inbreeding coefficient (F_{IS}), and little change in the fixation index (F_{ST}) between laboratory populations, suggest that genetic drift processes were not determinant in comparison to selection during the evolution experiment.

11. RNA-sequencing and subsequent bioinformatics analyses revealed a large number (3,068) of differentially expressed genes (DEGs) in the comparisons between selective regimes (predictable vs unpredictable), diapause conditions (non-forced vs forced) and their combinations.

12. Out of the 3,068 DEGs identified, 2,900 DEGs were found in the comparisons between the two contrasting selective regimes for both diapause conditions. This suggests that the selective regime is more important in driving differences in the transcriptome profile of diapausing eggs than the diapause condition assayed.

13. Most of the DEGs (2,815) were found to be up-regulated in the diapausing eggs produced under the predictable regime. These

genes could be related to the reactivation of the embryo and hatching readiness, since diapausing eggs produced under the predictable regime showed earlier and higher hatching fractions.

14. Genes related to the maintenance and termination of diapause were differentially expressed in *B. plicatilis* diapausing eggs produced under the two contrasting regimes of environmental fluctuation (predictable vs unpredictable).

Resum en Valencià

Introducció

Els organismes habiten entorns en què les condicions abiòtiques i biòtiques no són constants, sinó que varien tant en l'espai com en el temps. Aquestes fluctuacions ambientals són de naturalesa ubiqua i suposen fortes pressions de selecció a les quals els organismes han de respondre de manera adaptativa. En conseqüència, conéixer com els organismes responen per fer front a les fluctuacions ambientals s'ha convertit en un tema central en ecologia evolutiva.

L'heterogeneïtat ambiental, tant temporal com espacial, és un factor clau en l'evolució de la dispersió. La dispersió és un mecanisme que permet als organismes viatjar tant en l'espai com en el temps per escapar de les condicions adverses. En alguns organismes la dispersió s'ha associat amb la producció de formes de resistència inactives que poden romandre latents durant llargs períodes de temps. Aquestes formes latents representen una adaptació molt important que es produeix en les històries vitals de molts organismes per tal d'evitar l'extinció de les poblacions i promoure la dispersió i colonització de nous habitats.

La diapausa és un tipus de dormició, que es caracteritza per una suspensió temporal del desenvolupament i de l'activitat metabòlica, i que ofereixen una major resistència a les condicions

ambientals adverses. En funció de l'espècie o les condicions ambientals, la diapausa es pot produir en diferents etapes del cicle vital de l'organisme (per exemple; ous, larves, pupes o adults). Tant l'entrada en diapausa, com la seua duració, depèn d'un control fisiològic intern, i per tant, pot perdurar inclús quan les condicions favorables es restableixen. Aquestes formes que no desclouen es mantenen viables al sediment contribuint a la formació dels anomenats "bancs d'ous diapausics". Aquests bancs d'ous actuen com a reservori de diversitat genètica i poden promoure tant la persistència de les espècies, com el manteniment de la variabilitat fenotípica i genètica entre les poblacions. És per això que la diapausa ha sigut considerada com una estratègia per evitar riscos, treballant com una mena de "màquina del temps".

Aquesta tesi es centra en les fluctuacions ambientals temporals, que es caracteritzen per l'alternança entre condicions ambientals favorables i adverses. Aquesta alternança es pot considerar predictible, si es produeix de forma recurrent; o impredictible, si l'alternança segueix un patró aleatori. En aquest sentit, la diapausa pot ser objecte de diferents respostes adaptatives als organismes que habiten ambients amb fluctuacions temporals. Una primera forma de resposta per fer front a la variació ambiental és la plasticitat fenotípica, mitjançat la qual un genotip pot produir diferents fenotips en funció de condicions particulars de l'ambient. Aquesta resposta s'espera que ocórrega en entorns que fluctuen

predictiblement i quan els canvis ambientals no són massa ràpids. Quan les fluctuacions ambientals es produeixen lentament, un segon tipus de resposta anomenada *adaptive tracking* s'espera que evolucione. En aquest tipus de resposta la selecció natural actua recurrentment sobre la variació hereditària que existeix entre individus i condueix a l'evolució genètica. Per últim, si el canvi ambiental és ràpid i impredictible s'espera que evolucione l'anomenat *bet hedging*, una adaptació en la història vital de minimització dels riscs. El *bet hedging* redueix la variació temporal en l'eficàcia biològica quan hi ha alta variància ambiental. El *bet hedging* s'ha considerat majoritàriament com a una estratègia materna i s'han descrit dos modes principals. Un primer mode, l'anomenat *bet hedging* conservatiu, es produeix quan un únic genotip produeix en la seua descendència un únic fenotip de baix risc per tal de reduir la variància temporal en l'èxit reproductiu. Un segon mode, anomenat *bet-hedging* diversificador, es produeix quan un únic genotip produeix diversos fenotips en la seua descendència. Ambdós tipus de *bet hedging* són aparentment respostes subòptimes quan es considera la mitjana de les condicions ambientals, però poden augmentar l'eficàcia biològica de l'organisme si es considera el llarg termini. Tot i que el *bet hedging* ha estat ben desenvolupat teòricament en l'ecologia evolutiva, l'evidència empírica encara és escassa.

En les poblacions naturals, la impredictibilitat ambiental pot actuar sobre diversos trets de la història vital d'un organisme, especialment en aquells que tenen cicles de vida complexos. La diapausa és un exemple clàssic d'un important component de la història vital en el qual diferents trets poden ajudar a fer front a la impredictibilitat de l'habitat. Un d'aquests trets és el moment d'entrar en diapausa. El moment de passar de les etapes actives a les etapes diapàusiques (inactives) és un component crític de l'eficàcia biològica. La producció de formes diapàusiques hauria de produir-se abans del començament de condicions inadequades, ja que aquestes formes de resistència són essencials per a la futura supervivència d'un genotip. L'assignació de recursos per produir les formes de diapausa comporta costos per als organismes, ja que implica una menor inversió en el creixement de la població. Tanmateix, si les condicions favorables es mantenen, una inversió prematura en diapausa pot ser poc òptima, ja que es redueix un potencial major creixement de la població. També es produeixen costos similars pel que fa a un altre tret relacionat amb la diapausa: el moment de sortir de diapausa. En condicions ambientals favorables, sortir de diapausa pot afavorir l'explotació dels recursos ambientals i, per tant, millorar la capacitat competitiva dels organismes reactivats. Tanmateix, abandonar la diapausa pot suposar un risc si l'hàbitat es torna inesperadament advers i els

genotips no tenen temps suficient per a produir una nova cohort de formes diapàusiques.

L'evolució experimental pot ser una eina útil per estudiar les respostes adaptatives a les fluctuacions ambientals. A la fi del segle XX, Stephen Jay Gould en el seu llibre *Wonderful life* (1989) va popularitzar la idea d'un experiment hipotètic anomenat "replaying life's tape". Aquest experiment mental implica d'alguna manera rebobinar la història de la vida al seu punt de partida inicial per tal de saber si la història tornaria a repetir-se. Aquesta idea s'ha associat als estudis d'evolució experimental com a forma de provar la reproductibilitat dels resultats evolutius. L'essència de l'evolució experimental és l'estudi dels processos evolutius que ocorren en poblacions de laboratori i de camp en resposta unes condicions imposades per l'experimentador. Aquest enfocament ha estat reconegut com una eina valuosa en l'ecologia i l'evolució per (1) detectar respostes evolutives a curt termini, i (2) explorar les bases genòmiques de l'adaptació. Aquesta aproximació experimental posseïx una sèrie d'avantatges per al seguiment de les trajectòries evolutives com són que les poblacions de laboratori evolucionen sota condicions controlades (és a dir, amb pressions selectives definides), i a més a més que es poden realitzar repliques en cadascun dels règims selectius fixats. Aquests tipus d'estudis solen comparar grups de poblacions derivades del mateix genotip ancestral, que s'exposen a diferents règims selectius al llarg del

temps. Els genotips ancestrals, les poblacions o els individus que s'utilitzen per establir les poblacions experimentals, són clau per comprendre els processos evolutius que es poden produir al llarg de l'assaig d'evolució experimental. Si la població fundadora és genèticament uniforme, l'adaptació als règims selectius podria donar-se per l'acumulació de mutacions beneficioses. En canvi, si la població fundadora és polimòrfica, s'espera que la selecció actue en la variació hereditària. Es preveu que aquest últim tipus de selecció produïsca una evolució ràpida en hàbitats nous. Per tant, l'evolució experimental té el potencial de mostrar l'adaptació dels organismes, en un nombre reduït de generacions, a diverses pressions de selecció i s'ha utilitzat àmpliament en un rang d'organismes filogenèticament ampli. Entre els metazous, hi ha diversos estudis que han aplicat amb èxit aquest enfocament per abordar l'adaptació a les fluctuacions ambientals. Curiosament, els rotífers monogononts han estat reconeguts recentment com a organismes adequats per a provar hipòtesis evolutives, a més a més d'haver-se utilitzat amb èxit respecte a diversos factors biòtics i abiòtics. Diverses característiques converteixen a aquests rotífers en un model excel·lent per als estudis d'evolució experimental, com (1) la seua reduïda grandària (normalment < 1 mm), que permet establir tamanys poblacionals elevats, (2) el seu curt temps de generació, i (3) el fet que es cultiven fàcilment al laboratori. A més a més, els rotífers són, fins ara, els únics metazous capaços d'assolir

un creixement demogràfic constant en cultius continus. En aquest tipus de cultius, el medi fresc es renova constantment a un ritme fixe, permetent que els organismes es puguen cultivar en un estat fisiològic constant baix condicions controlades. A més de les característiques esmentades anteriorment, els rotífers tenen (4) un cicle de vida complex que combina la reproducció sexual i asexual. La reproducció asexual permet establir fàcilment llinatges clonals i, per tant, utilitzar genotips replicats en experiments. En canvi, la reproducció sexual produeix noves variants genètiques en poblacions experimentals, la qual cosa permet a la selecció actuar. Finalment, en aquests rotífers (5) s'ha descrit una superposició entre les escales de temps evolutives i ecològiques, el que demostra el seu potencial per estudiar les retroalimentacions eco-evolutives.

El rotífer monogonont *Brachionus plicatilis* és un habitant comú d'aigües continentals, salines i salobres. Aquests hàbitats sovint es caracteritzen per fortes fluctuacions a la longitud de l'estació de creixement (és a dir, el període de temps en què les poblacions de rotífers estan actives a la columna d'aigua). Per tant, l'hàbitat es pot convertir en advers de forma impredictible. Aquests patrons de fluctuació són típics dels cossos de l'aigua de la regió mediterrània, els quals presenten una àmplia gamma de predictibilitat ambiental. *B. plicatilis* té un tipus de reproducció anomenada partenogènesi cíclica, en què la proliferació per partenogènesi ameiòtica (fase asexual) es combina amb episodis ocasionals de producció de

mascles i reproducció sexual (fase sexual). Les poblacions actives a la columna d'aigua estan constituïdes principalment per femelles asexuals que produeixen filles genèticament idèntiques a les seves mares mitjançant partenogènesi. Es tracta d'una espècie de propagació clonal que permet un creixement ràpid de la població, permetent-los colonitzar nous hàbitats mitjançant una o unes poques femelles fundadores. Després d'aquest període inicial de propagació clonal: durant la qual la població està formada per llinatges clonals, la reproducció sexual es veu induïda per un senyal químic produït pels rotífers. Aquesta substància química s'acumula al medi quan augmenta la densitat de la població. Una vegada que s'aconsegueix un llindar de densitat poblacional, les femelles asexuals passen a produir filles sexuals en una fracció de la seua descendència. Per tant, la reproducció sexual i asexual estan superposades. Les femelles sexuals produeixen ous haploides que es desenvolupen tant en mascles o, si es fertilitzen, en ous de diapausa (diploides). Aquests ous de diapausa són embrions resistents i inactius, que s'enfonsen i s'acumulen en els sediments on romanen inactius durant un període de temps variable. Quan les condicions de l'hàbitat són favorables, s'indueix la desclosa dels ous de diapausa donant lloc a noves femelles asexuals que recolonitzen la columna d'aigua, començant una nova estació de creixement. No obstant això, no tots els ous de diapausa desclouen en l'estació de creixement següent a la seua producció. Aquests ous sovint

mostren diapausa prolongada i s'acumulen als sediments formant els anomenats bancs d'ous diapàusics. Aquesta estratègia ha sigut considerada com una estratègia de minimització de riscs en ambients amb fluctuacions impredictibles, ja que són essencials per a la persistència a llarg termini de les poblacions rotífers. En conjunt, el cicle de vida del rotífer monogonont es considera una adaptació a entorns temporalment diferents.

Desentranyar els mecanismes moleculars i la base genòmica subjacent a l'adaptació dels organismes als seus ambients són temes clau en biologia evolutiva. En els últims anys, els avanços moleculars i tecnològics juntament amb els costos decreixents en la seqüenciació de nova generació (*Next-generation sequencing*; NGS) han revolucionat l'anomenada "època òmica". Aquest nou context científic ha permès analitzar una gran quantitat de dades tant d'espècies model com d'aquelles que no ho són. No obstant això, els recursos genòmics en organismes que no són model encara són escassos i per tant, la capacitat de descobrir gens funcionalment rellevants és limitada. Una de les tecnologies "òmiques" més utilitzades és la genòmica, que es centra en la comprensió de l'estructura, la funció i l'evolució dels genomes. Mitjançant la caracterització de la variació genòmica, les tecnologies genòmiques permeten avaluar l'estructura de la població i identificar els genotips que poden ser responsables de les adaptacions. Una altra tecnologia "òmica" àmpliament utilitzada és

la transcriptòmica, que es basa en l'estudi de gens que s'expressen activament en un moment donat o en associació a una condició (o un conjunt de condicions) d'interès. En conjunt, aquestes tècniques "òmiques" permeten identificar els gens candidats que poden ser responsables de les adaptacions en els trets de la història vital i, per tant, promouen la integració entre dades genètiques i ecològiques.

Objectius

Aquesta tesi aprofita els avanços tècnics abans esmentats i adopta el marc teòric en ecologia evolutiva per abordar l'estudi de les respostes adaptatives als ambients impredictibles de les poblacions de rotífers utilitzant una aproximació d'evolució experimental. Els principals objectius d'aquesta tesi són: (**1**) Estudiar la resposta adaptativa de trets de la història vital relacionats amb la diapausa en poblacions del rotífer *B. plicatilis* sotmeses a dos règims selectius fluctuants (predictibles vs impredictibles) simulats al laboratori, (**2**) Dilucidar la base genòmica de l'adaptació a la impredictibilitat de l'hàbitat en les poblacions de *B. plicatilis* evolucionades en els dos règims divergents, utilitzant tecnologies genòmiques, i (**3**) explorar l'expressió genètica dels ous de diapausa produïts per les poblacions de rotífers que van evolucionar sota aquests dos règims selectius.

Esquema de la tesi: metodologia i resultats principals

La tesi, en les parts següents a la introducció general (**Capítol 1**), està organitzada de la manera següent.

El **capítol 2** ofereix una visió general de la metodologia utilitzada en el desenvolupament dels objectius de la tesi. En primer lloc, es descriuen les principals característiques biològiques de l'espècie model *B. plicatilis*. En segon lloc, s'introdueixen les aproximacions d'evolució experimental i es proporciona una visió general sobre la seua utilitat en els estudis d'ecologia i evolució. En tercer lloc, s'explica el disseny de l'evolució experimental utilitzat al llarg d'aquesta tesi, amb dades sobre l'establiment de poblacions de laboratori de *B. plicatilis*. També s'indica com es van sotmetre a aquestes poblacions als dos règims de fluctuació (predictibles vs impredictibles) durant vuit cicles de selecció, i com van ser interromputs cadascun dels cicles per períodes d'adversitat. Breument, en aquest experiment, tres poblacions de laboratori (que actuen com a repliques) es van assignar aleatòriament al règim de selecció predictible, caracteritzat per estacions de creixement (és a dir, hidroperíode) de durada constant (28 dies). Les altres tres poblacions de laboratori es van assignar a un règim de selecció impredictible que es va caracteritzar per estacions de creixement d'una longitud que variava a l'atzar, però que tenien una mitjana de duració de 28 dies. Amb aquest disseny, es pot suposar que aquests règims de selecció són predictibles i impredictibles,

respectivament, pels rotífers. Cal remarcar que aquest disseny imita la selecció imposada per la impredictibilitat de l'hàbitat en les poblacions de rotífers naturals. Finalment, en aquest capítol es descriuen les principals tècniques moleculars utilitzades al llarg de la tesi (*Genotyping by sequencing*, d'ara endavant GBS i de seqüenciació d'ARN, d'ara endavant RNA-Seq).

En el **capítol 3**, es va estudiar l'adaptació a ambients impredictibles a través d'una aproximació d'evolució experimental utilitzant el rotífer *B. plicatilis* com a organisme model. L'objectiu d'aquest capítol fou testar com els ambients impredictibles podien seleccionar trets relacionats amb la diapausa en poblacions del rotífer *B. plicatilis*. Es van estudiar dos trets de la història vital relacionats amb la diapausa en poblacions evolucionades sota els dos règims selectius (predictibles vs impredictibles) durant el desenvolupament de l'experiment d'evolució. En hàbitats impredictibles, la supervivència de les poblacions de rotífers depèn dels ajustaments dels seus trets de la història vital a la variació en la durada de les estacions de creixement. Donat que la diapausa és l'única forma de sobreviure a condicions inadequades entre les estacions de creixement, almenys hi ha dos trets relacionats amb la diapausa que es poden considerar claus: (1) el moment d'inici del sexe (una aproximació a l'inici de la producció d'ous de diapausa), i (2) la fracció de desclosa d'ous de diapausa (inversament relacionada amb la durada de la diapausa). Estudis teòrics han

proposat el moment d'inici del sexe com una estratègia de *bet-hedging* conservatiu en poblacions de rotífers que habiten ambients impredictibles. Si el final de l'estació de creixement no es pot preveure, aleshores començar la reproducció sexual com més aviat millor pot evitar el risc d'estacions de creixement inesperadament breus que poden provocar l'extinció del genotip. No obstant això, iniciar el sexe massa prompte quan encara les condicions del hàbitat són favorables, redueix l'eficàcia biològica, ja que la inversió en sexe (i la producció de ous de diapausa) resulta en una reducció directa de la taxa de creixement poblacional. Per contra, la fracció de desclosa d'ous de diapausa s'ha proposat en diversos estudis teòrics com una forma de *bet-hedging* diversificat (duració de la diapausa variable en ous produïts per un mateix genotip). D'acord amb la teoria, en hàbitats predictibles, s'espera una tassa elevada de desclosa d'ous de diapausa en l'estació de creixement següent a la seva producció. D'aquesta manera, els individus que desclouen podrien produir una nova cohort d'ous de diapausa durant l'estació de creixement, i els bancs d'ous serien renovats contínuament entre les successives estacions de creixement. En lloc d'això, als hàbitats impredictibles, si tots els ous desclouen i es produeix una estació de creixement inesperadament curta, no es produiria cap augment en el banc d'ous. Per tant, si tan sols una fracció dels ous de diapausa desclouen, un genotip de rotífer assegurarà la seva presència en períodes posteriors

favorables i, per tant, evitarà el risc d'extingir-se. Durant l'experiment d'evolució, es van caracteritzar dos trets de la història vital en cada règim selectiu. Es va estudiar el moment de inici del sexe realitzant 720 bioassaigs (4 cicles de selecció × 6 poblacions × 10 clons × 3 rèpliques). La resposta evolutiva en la fracció de desclosa d'ous de diapausa es va estudiar mitjançant la realització de més de 20,000 bioassaigs (4 cicles × 96 ous de diapausa × 10 clons × 6 poblacions). Ambdós trets es van estimar sobre el mateix conjunt de clons. Les poblacions de laboratori de rotífers van mostrar una adaptació ràpida a ambients impredictibles, mostrant una resposta divergent en aquests dos trets de la història vital. Les poblacions sotmeses al règim de selecció impredictible mostraren una menor fracció de desclosa d'ous de diapausa i un inici de sexe més prematur. Aquests resultats suggereixen que les estratègies de *bet hedging,* o estratègies per evitar riscs, són la base de l'adaptació a la impredictibilitat ambiental en aquests organismes.

En el **capítol 4**, les bases genòmiques de l'adaptació a la impredictibilitat ambiental es van desentranyar a les mateixes poblacions de laboratori que es van utilitzar al capítol 3. Mitjançant GBS, es van identificar polimorfismes genètics en 169 clons provinents d'ambdós règims de selecció després del setè cicle de l'experiment d'evolució. Aquestes dades es van comparar amb dades de GBS publicades de 270 clons de camp de rotífer, els quals es van utilitzar per establir les poblacions de laboratori. Com a

resultat de l'anàlisi de dades de GBS, es van identificar 6,107 polimorfismes de nucleòtid simple (SNPs). Aquests SNPs es van utilitzar per identificar els loci més atípics i, per tant, per identificar-los com a candidats a estar sota selecció respecte a la impredictibilitat ambiental. Hi ha diversos mètodes que tenen com a finalitat la detecció de loci sota selecció. Entre ells, els mètodes basats en F_{ST} comparen les freqüències al·lèliques entre les poblacions per identificar aquells SNP que tenen valors de diferenciació més alts que els esperats sota un model d'evolució neutral. Més recentment, s'ha desenvolupat un altre grup de mètodes com el GWAS (*genome wide association studies*) per tal de detectar signatures de selecció. Aquests mètodes es basen en l'associació de les variacions fenotípiques amb la corresponent variació genotípica a nivell d'individu. Després d'aplicar aquestes anàlisis, es van identificar 76 SNPs candidats a estar sota selecció. Tres d'ells van canviar les seves freqüències al·lèliques en resposta al règim de selecció impredictible, convertint-se en candidats a estar sota selecció en ambients impredictibles. La resta de SNPs van experimentar canvis paral·lels en les freqüències al·lèliques –respecte a la població original– en totes les poblacions de laboratori, la qual cosa suggereix una adaptació a les condicions de laboratori. Addicionalment, una anàlisi d'associació de genotip-fenotip va revelar cinc SNP associats amb els dos trets de la història vital relacionats amb la diapausa. Quatre d'aquests cinc SNPs

(ubicats en dos gens diferents) es van associar a la fracció de desclosa d'ous de diapausa, i l'altre SNP es va associar al moment d'inici del sexe. En general, les poblacions de laboratori sotmeses als dos règims de selecció van mostrar valors més baixos de F_{ST} que les poblacions naturals originaries. D'altra banda, la diversitat genètica es va mantenir durant tot l'experiment en totes les poblacions de laboratori, excepte en una població del règim impredictible en la qual es va observar una lleugera disminució de la diversitat genètica. No obstant això, (1) la pèrdua insignificant de la diversitat genètica, (2) els baixos valors del coeficient d'endogàmia i (3) el petit canvi en F_{ST} entre poblacions de laboratori suggereixen que els processos de deriva genètica no han sigut determinants en comparació amb els processos associats a la selecció.

En el **capítol 5** es va realitzar una anàlisi de transcriptòmica de les dites poblacions de rotífers experimentals per estudiar els mecanismes moleculars associats a la diapausa en resposta a la impredictibilitat ambiental. L'objectiu principal d'aquest capítol era identificar i quantificar l'expressió dels gens implicats en (1) el manteniment de la diapausa, i (2) la sortida d'ella en *B. plicatilis*. Per dur a terme aquest estudi, després del vuitè cicle de selecció, es van recollir 10,000 ous de diapausa de cada població de laboratori i es van dividir en dos grups per ser sotmesos a condicions que promovien o bloquejaven la desclosa. L'expressió gènica es va

analitzar mitjançant RNA-seq. Després d'aplicar un conjunt d'anàlisis bioinformàtics, es van identificar un total de 3,068 gens expressats diferencialment en ous de diapausa de rotífers. Les anàlisis realitzades en aquest capítol revelen que els gens relacionats amb el manteniment de la diapausa i la seva terminació presenten una expressió diferencial en ous de diapausa produïts en un règim de selecció predictible respecte als produïts en el règim impredictible. Aquest estudi amplia el coneixement dels complexos esdeveniments moleculars i cel·lulars que es produeixen durant la diapausa. Alguns dels gens identificats en aquest estudi són ben coneguts en altres organismes que presenten formes de resistència, però alguns d'ells són nous i cal investigar més per a determinar el seu paper en la dessecació i la tolerància a l'estrès.

Finalment, el **capítol 6** ofereix una discussió general sobre els principals resultats obtinguts en aquesta tesi, proposa una investigació prospectiva i exposa les conclusions més importants.

Conclusions

Les principals conclusions derivades d'aquesta tesi s'enumeren a continuació:

1. Les poblacions del rotífer *Brachionus plicatilis* van evolucionar ràpidament produint respostes adaptatives a la impredictibilitat ambiental durant l'evolució experimental.

2. Els trets relacionats amb la diapausa, el moment de l'inici del sexe i la fracció de desclosa d'ous de diapausa, van evolucionar de forma divergent en les poblacions de laboratori sotmeses als dos règims de selecció fluctuants (predictible vs impredictible).

3. L'inici del sexe va ser més prematur en les poblacions de laboratori sotmeses al règim impredictible. Aquesta resposta suggereix l'existència d'una estratègia de *bet hedging* conservatiu per tal de proporcionar protecció contra les estacions de creixement inesperadament curtes.

4. Les poblacions de rotífers que van evolucionar en el règim de selecció impredictible presentaren una duració de la diapausa més llarga (és a dir, fraccions de desclosa més baixes) que les poblacions evolucionades en el règim predictible. Això suggereix l'evolució de respostes de *bet hedging* diversificadores, que promouen períodes de diapausa més llargs i afavoreixen la supervivència de les poblacions de rotífers per la seva persistència en els bancs d'ous de diapausa quan l'ambient és impredictible.

5. S'ha trobat una desclosa d'ous de diapausa asincrònica en les poblacions evolucionades en el règim predictible. Això podria interpretar-se com una aposta de dispersió de riscs dins de l'estació de creixement per aquest tret. Aquesta estratègia podria evolucionar si la ocurrència d'estacions de creixement és predictible, però hi ha certa incertesa respecte al seu inici.

6. El *Genotyping by sequencing* (GBS) i l'anàlisi bioinformàtic posterior proporcionaren un gran nombre (6,107) de polimorfismes de nucleòtid simple (SNP) d'alta qualitat.

7. Tres SNPs –localitzats en tres gens diferents– van mostrar una diferenciació genètica més alta entre els règims de selecció de la qual s'espera per atzar. Per tant, són candidats a estar sota selecció en ambients impredictibles.

8. Les anàlisis de genotip i fenotip van revelar quatre SNPs candidats a estar associats a la fracció de desclosa d'ous de diapausa i un SNP associat al moment de l'inici del sexe.

9. Els canvis paral·lels en freqüències al·lèliques de diversos SNP candidats a estar sota selecció es van trobar en les sis poblacions de laboratori, independentment del seu règim de selecció. Això suggereix un senyal fort d'adaptació a les condicions de laboratori durant l'evolució experimental.

10. La pèrdua insignificant de la diversitat genètica, els baixos valors del coeficient d'endogàmia (F_{IS}) i els reduïts canvis en l'índex de fixació (F_{ST}) en les poblacions de laboratori suggereixen que els processos de deriva genètica no són determinants en comparació amb el paper de la selecció durant l'experiment d'evolució.

11. La seqüenciació d'ARN i les posteriors anàlisis bioinformàtiques revelaren un gran nombre (3,068) de gens expressats diferencialment en les comparacions tant entre els dos règims de

selecció (predictibles vs impredictibles), com en les condicions de diapausa (no forçades i forçades) i en les seves combinacions.

12. Dels 3,068 gens expressats diferencialment, 2,800 es van trobar en les comparacions entre els dos règims selectius en les dues condicions de diapausa. Això suggereix que el règim de selecció té un efecte més important en el perfil transcriptòmic dels ous de diapausa que les condicions de diapausa assajades.

13. La majoria dels gens amb expressió diferencial (2,815) es van trobar sobre expressats en ous de diapausa produïts en el règim predictible. Aquests gens podrien estar relacionats amb la reactivació de l'embrió i la predisposició per la desclosa, ja que els ous de diapausa produïts sota el règim predictible també van presentar una major i més prematura desclosa.

14. Gens relacionats amb el manteniment i la terminació de la diapausa es van trobar diferencialment expressats en els ous de diapausa de *B. plicatilis* produïts sota els dos règims de fluctuació ambiental (predictible vs impredictible).

1

Introduction

The evolutionary challenge of environmental heterogeneity

Organisms inhabit environments whose abiotic and biotic conditions are not constant, but vary temporally and/or spatially (Kolasa and Rollo 1991; Vasseur and McCann 2007; Pearman et al. 2008). These environmental fluctuations are ubiquitous in nature and impose strong selection pressures to which organisms must adaptively respond (Levins 1968; Meyers and Bull 2002; Botero et al. 2015). The importance of environmental fluctuations for evolutionary and ecological processes has been proved in many different frameworks, including population dynamics (e.g., Lewontin and Cohen 1969; Yoshimura and Jansen 1996; Lande et al. 2009), life-history evolution (e.g., Wilbur et al. 1974; Hastings and Caswell 1979), dispersal (e.g., Southwood 1962; Roff 1974; Levin et al. 1984), foraging behavior (e.g., Chesson 1978; Schmickl and Crailsheim 2004), patterns of natural selection (e.g., Frank and

Slatkin 1990; Chevin et al. 2010; Melbinger and Vergassola 2015), coexistence of species (e.g., Shorrocks et al. 1979; Chesson 1986), predation (e.g., Huffaker 1958; Dobramysl and Täuber 2013), and species diversity maintenace (e.g., Rashit and Bazin 1987; Chesson 2000; Petchey et al. 2002; Borrvall and Ebenman 2008). Consequently, unveiling the adaptive response of organisms to cope with heterogeneous environments has become a central topic in fundamental and applied evolutionary ecology (e.g., Meyers and Bull 2002; Parmesan 2006; Lenormand et al. 2009; Simons 2011).

Environmental heterogeneity can be understood as the sum of spatial heterogeneity –where biotic and abiotic ecological factors vary across space (e.g., patchiness)– and temporal heterogeneity –in which organisms are challenged by temporal changes in ecological factors within a generation or between generations– (Pigliucci 2001). Spatially heterogeneous environments are composed of patches of suitable and unsuitable habitats. The latter are habitats with inhospitable or poor environmental conditions in which organisms are not able to survive or reproduce (i.e., suitability is defined in terms of fitness; Southwood 1977). In addition, if the environment is temporally heterogeneous, fluctuation could cause some habitat patches become temporally unsuitable, thus leading to an alternation between suitable and unsuitable conditions. Both, spatial and temporal heterogeneity were considered by Southwood (1977; 1988) to classify the habitats

in distinct categories, which provides a frame that determines the evolution of life histories. The environmental regimes of spatial heterogeneity (adversity) and temporal heterogeneity (disturbance) were regarded by this author as constituting a "habitat templet" that constrains the types of traits appropriate for local persistence of populations (Townsend and Hildrew 1994). Thus, spatial and temporal features of the environment may determine the type and range of ecological adaptive responses (see below).

Temporal and spatial heterogeneity of the environment is a key factor in the evolution of dispersal (McPeek and Holt 1992; Ronce 2007; Starrfelt and Kokko 2012b), which is a central life-history strategy that ultimately causes gene flow through space or time (Gibbs et al. 2010). To escape from unsuitable environmental conditions, organisms can disperse either in space or time (Venable and Lawlor 1980; Starrfelt and Kokko 2012b; Buoro and Carlson 2014). *Dispersal in space* can be driven by an active movement of the organisms (e.g., active dispersal) or by means of environmental forces, such as water flow, wind or other organisms (i.e., passive dispersal). The latter is related to the production of dormant, resistant stages in some species (e.g., seeds in plants, diapausing eggs in zooplankton). Indeed, these resistant forms are not only associated to dispersal in space, since the delay in the recruitment of an individual to a population caused by dormancy may be

regarded as a type of *dispersal in time* (Levin et al. 1984; Venable and Brown 1988; Vitalis et al. 2013; García-Roger et al. 2014). Consequently, these resistant forms constitute a successful way to escape from temporally unsuitable conditions. Moreover, besides to "travel in space" and to "travel in time", some organisms (i.e., facultatively sexual organisms) can face unsuitable conditions by switching between asexual and sexual reproduction. The production of new genetic variants by sexual reproduction can increase offspring fitness (Griffiths and Bonser 2013) and allows organisms to tolerate new conditions. This third option –to "travel in identity" (*sensu* Gerber 2018)– constitutes a new perspective of sexual reproduction based on the so-called "abandon-ship hypothesis". This hypothesis predicts higher allocation to sexual reproduction under stressful environmental conditions, allowing low-fitness individuals to recombine their genotype and therefore to increase their offspring fitness (Hadany and Otto 2007; 2009; Griffiths and Bonser 2013).

Dormant stages and dispersal in time

The production of resistant, dormant stages is a major and widespread adaptation that occurs in the life histories of many organisms (i.e., from bacteria to animals; Alekseev et al. 2012). Dormant stages promote dispersal strategies –both in time and

space– either to anticipate or to respond to environmental changes (Baumgartner and Tarrant 2016).

Dormancy is a relative inactive state in an organism's life cycle during which metabolic activity and growth are minimized, and development is temporarily arrested. During dormancy, the organism saves energetic resources, which can be used later when activity resumes at the onset of favorable conditions. Another advantage is that dormant stages are often resistant to unsuitable environmental conditions (i.e., drought, temperature and salinity extremes, low dissolved oxygen, lack of light, etc.; e.g., Begon et al. 2006; Hairston and Fox 2009).

A variety of forms of dormancy differing in the intensity and duration of this inactive stage have been described (see Lee 2009). Among animal phyla, the two main forms of dormancy are quiescence and diapause (Cáceres 1997). Quiescence arises under direct impact of unsuitable environmental conditions (Ricci 2001; Alekseev et al. 2012; Poelchau et al. 2013). When suitable conditions are reestablished, development and/or activity is resumed. Then, this type of dormancy is controlled by single external factors such as temperature, photoperiod, oxygen, or a combination of them (Danks 1987; Brendonck 1996). By contrast, both the initiation and the duration of diapause depend on endogenous control –i.e., they are mostly regulated by internal

physiological factors– (Brendonck 1996; Hand and Podrabsky 2000). Before the occurrence of adverse conditions, a signal indicating that conditions will soon deteriorate (e.g., day-length, temperature, food concentration, population density, etc.; Hairston and Fox 2009) triggers organisms to initiate diapause. This requires a preparatory phase that typically precedes the onset of the unfavorable conditions (Baumgartner and Tarrant 2016). Moreover, the developmental arrestment of diapause remains even if suitable conditions resume, until specific cues disrupts it (Tauber et al. 1986; Hairston et al. 1995; Cáceres 1997). Despite being both a metabolic and development arrestment, diapause does not imply a simple shutting down of the genome. Instead, several studies have shown that there are genes that are specifically up-regulated or expressed intermittently during diapause (Denlinger 2002; Van Straalen and Roelofs 2011; see Chapter 5). This type of dormancy is widespread in the animal kingdom (Danks 1987). Depending on the species and the type of habitat, diapause can occur at different moments of the life cycle (e.g., egg, larva, pupa or even adult in copepods and insects; Ricci and Pagani 1997; Gordon and Headrick 2001; Fan et al. 2013; Baumgartner and Tarrant 2016; Diniz et al. 2017).

Many diapausing stages (e.g., seeds, eggs and spores) do not germinate or hatch when exposed to suitable environmental conditions, so that they accumulate in the sediment forming long-

lived banks of propagules (Brock et al. 2003), commonly called "seed banks" or "egg banks". These diapausing-stage banks can persist for extended periods of time (Gilbert 1974; Boulton and Lloyd 1992; Brock 1998), favoring both the dispersal in time and in space (Venable and Brown 1988; Hairston 1998; Schröder 2005). Given that these resistant forms are produced under different biotic and abiotic conditions, the diapausing-stage banks act as genetic reservoirs of genotypes adapted to multiple environmental conditions (Hairston 1996; Ortells et al. 2000; Brendonck and De Meester 2003; Montero-Pau 2012). All this allows the maintenance of both phenotypic and genetic variability within populations.

Diapause and adaptive responses to environmental unpredictability

There are a variety of adaptive responses that organisms can develop to overcome the unsuitable conditions caused by temporal and spatial environmental heterogeneity. In the case of temporally varying environments, adaptation depends on: (1) the graininess of environmental variation –i.e., how rapidly the environment changes within the lifetime of an individual (Levins 1968)–, for instance, daily, seasonally, yearly, etc.; (2) the magnitude of the fluctuations that an organism typically experiences across its lifetime; and (3) the predictability of the environmental fluctuations

faced by the organisms, both within and across generations (Via et al. 1995; Meyers and Bull 2002). As mentioned above, temporally varying environments entail an alternation between suitable and unsuitable conditions. Such alternation can be characterized as predictable, if it occurs recurrently, or unpredictable, if the alternation occurs following a random pattern (Graham et al. 2014). However, the degree of predictability of environmental fluctuations does not only depend on the pattern of variation of suitable and unsuitable periods, but also whether this environmental fluctuation occurs within a time-scale relevant to the organism concerned. From the point of view of an organism, predictability is related to its ability to anticipate and adjust to a future environmental condition. Therefore, when studying adaptive responses to environmental unpredictability, the temporal scale of "environmental perception" of the organism of interest should be taken into account (Southwood 1977; Begon et al. 2006; Franch-Gras 2017a). Indeed, most natural populations experience environmental variation that is only partially predictable and which also might not be constant over time. There is not habitat feature that is entirely constant, or that changes in a perfectly predictable manner. Quantification of unpredictability is very relevant since its degree of prevalence in a habitat is expected to affect the adaptive responses of organisms (Franch-Gras et al. 2017a). Moreover, the adaptation of organisms to environmental unpredictability is

currently gaining researchers' attention due to the predicted scenario of increase in the intensity of environmental variation caused by climate change (IPCC 2013). Nonetheless, despite there are methodologies available to quantify the degree of predictability of habitat features (e.g., Colwell 1974; Sabo and Post 2008; Blanchet et al. 2008; Legendre and Gauthier 2014; Franch-Gras et al. 2017a), such quantification and the testing of adaptive responses under field conditions yet entail difficulties (see for instance Franch-Gras et al. 2017a). On one hand, quantifying predictability requires long time series of data –which are costly to obtain and often unavailable–, and most of the methodologies are very sensitive to gaps in the datasets (Legendre and Gauthier 2014). On the other hand, testing adaptive responses in natural populations – where populations are influenced by many biotic and abiotic ecological factors– is challenging because other correlated variables might be responsible for the observed response rather than the variable identified as variable of interest (e.g., Garland and Rose 2009; Kellerman et al. 2015). In this sense, the simulation of environmental unpredictability in laboratory experimental settings offers a valuable alternative, since it allows controlling for such confounding effects (see Chapters 2 and 6).

Diapause can be the subject of different adaptive responses in organisms inhabiting temporally varying environments (Simons et al. 2011; Botero et al. 2015), whose particular selective advantages

rely on (1) the degree of predictability, and (2) the relative timescale of environmental variation (Botero et al. 2015; Figure 1.1).

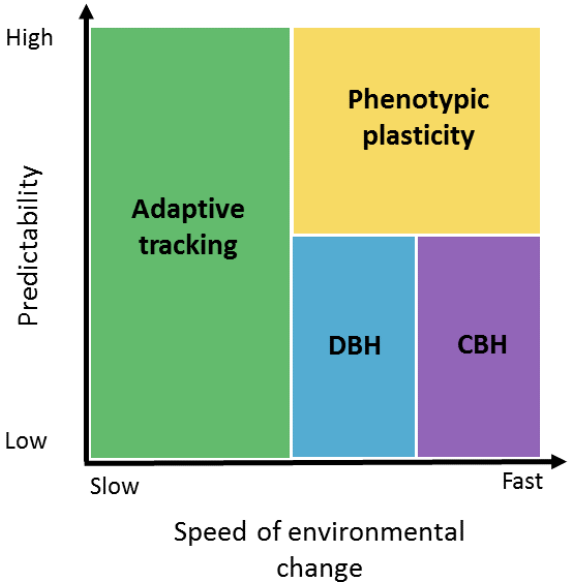


Figure 1.1. Expected adaptive responses in organisms inhabiting temporally varying environments regarding the degree of environmental predictability and the speed of the environmental change. CBH: conservative bet hedging; DBH: diversifying bet hedging. (Modified from Botero et al. 2015).

When environments fluctuate predictably –or, in general, when environmental cues are reliable (DeWitt and Scheiner 2004)– and changes do not occur too much rapidly, *phenotypic plasticity* is expected to be advantageous (Figure 1.1). However, this adaptive response –in which a single genotype produces different phenotypes depending on particular environmental conditions (Via

and Lande 1985; Reed et al. 2010)– will evolve if the phenotypic change is not too costly (Schlichting 1986; Stearns 1989; DeWitt and Scheiner 2004). In general, where environmental fluctuations occur slowly, a second type of adaptive response named *adaptive tracking* would be expected. In this response, natural selection acts recurrently on the standing heritable variation among individuals, which leads to genetic evolution (Lynch and Lande 1993; Tufto 2015). This adaptive evolution involves changes in the genetic frequencies of the population over generations that give rise to phenotypes that are more likely to persist in each new environment, thus allowing populations to adaptively track environmental changes. As stated above, this type of response requires the change to be slow, so that adaptation can take place before conditions change again. Finally, when environmental change is rapid and unpredictable –i.e., environmental cues are unreliable for the focus organism– selection in favor of *bet hedging* is expected to occur (Olofsson et al. 2009; Ripa et al. 2010). *Bet hedging* is a spreading-risk life-history adaptation displayed by individuals to reduce temporal variation in fitness –because of those occasional periods of especially low fitness– under conditions of unpredictable environmental variance (Cohen 1966; Olofsson et al. 2009; Ripa et al. 2010). It occurs when a selected trait in a genotype reduces fitness variance at the cost of a decrease in the arithmetic mean of fitness, in order to maximize geometric mean

fitness (i.e., the measure of long-term fitness; Seger and Brockmann 1987; Childs et al. 2010; Schreiber 2015). Bet hedging is in general conceived as a maternal strategy (Seger and Brockman 1987; Crean and Marshall 2009; Childs et al. 2010; García-Roger et al. 2014) with two main modes described in the literature (for review, see Childs et al. 2010). A first mode, called *conservative bet hedging*, takes place when a single genotype produces a unique low-risk phenotype in its whole offspring to reduce temporal variance in reproductive success (Philippi and Seger 1989). A second mode, *diversified bet hedging,* occurs when a single genotype produces diverse phenotypes among its offspring in advance of future unpredictable conditions. Both types of bet hedging are apparently suboptimal under the average environmental conditions but increase the long-term population growth rate (Simons 2011). Despite existing a large body of theoretical literature on the topic since the early development of bet-hedging theory by Cohen (1966) (see, for instance, Starrfelt and Kokko 2012a; Crowley et al. 2016; Schreiber 2015), empirical evidence of bet hedging is still scarce (Simons 2011; García-Roger et al. 2017).

The three adaptive responses described –phenotypic plasticity, adaptive tracking and bet hedging– are not mutually exclusive and may occur simultaneously in one organism. Firstly, because environmental variation can be broken down into predictable and unpredictable components (Crozier et al. 2008). Secondly, because

different features of the biology of an organism –as will be seen in the next section– can opt for one type of adaptive response or another (e.g., being more or less plastic, or protecting against risk) depending on the costs of the response (DeWitt and Scheiner 2004).

Environmental unpredictability and life-history traits related to diapause

One of the main objectives in ecology and evolution is to understand the variation of phenotypic features throughout the life cycle of an organism (e.g., growth, maturation, reproduction and survival; Coulson et al. 2006; Koons et al. 2016). These features –the so-called life-history traits– are considered relevant fitness components (e.g., Flatt and Heyland 2012). Life-history traits and life history strategies (i.e., combinations of the former) are traded-off (i.e., optimized) to maximize organism survival and reproductive success under different environments (Roff 1992; Stearns 1992; Flatt and Heyland 2012). Trade-offs play a central role in life-history evolution, and occur when organisms allocate resources to some life-history traits –e.g., growth, reproduction and survival– which are prioritized over others. Therefore, adaptation to a particular ecological condition occurs at the cost of being mal-adapted to other conditions. The evolution of life histories can be quantified

and analyzed using demography, quantitative genetics, mathematical modeling, and phylogenetic analyses (Flatt and Heyland 2012).

Unpredictability in natural populations can act on several life-history traits of an organism, especially in those having complex life histories (e.g., Crozier et al. 2008), and these traits could interact to reduce the impact of environmental variability (Brown and Venable 1986; Ellner et al. 1998; Childs et al. 2010). Diapause is a classic example of a major life history component enabling organisms to cope with environmental unpredictability (Hairston 1998; García-Roger et al. 2006).

Diapause encompasses different life-history trade-offs, which are expected to evolve in response to a suit of environmental conditions. In temperate zones, it has been described that the timing of switching from active to diapausing stages (i.e., entering diapause) is a critical component of fitness (Flatt and Heyland 2012). The production of diapausing stages needs to occur before the beginning of unsuitable conditions, since these stages are essential for the survival of the genotype (Brendonck and De Meester 2003; García-Roger et al. 2014; 2017). The assignation of resources to produce diapausing stages implies to lower the inversion on current population proliferation (Montero-Pau et al. 2014). However, an early investment in diapause can be sub-

optimal if suitable conditions remain, since the potential for further growth is reduced (Hairston and Munns 1984; Hairston and Olds 1984; Bradford and Roff 1993; Serra and King 1999; Simon et al. 2002; Roulin et al. 2013). Similar trade-offs happen with respect to the exit from diapause. Under suitable conditions, exit from diapause favors the exploitation of environmental resources and enhances the competitive ability of hatchlings (Alekseev et al. 2006; Gilbert 2012). However, exit from diapause can be risky if the habitat becomes unexpectedly unsuitable and the genotypes have not enough time to produce a new cohort of diapausing stages (Seger and Brockman 1987; Brendonck and De Meester 2003; Montero-Pau 2012; García-Roger et al. 2017; Franch-Gras 2017).

These trade-offs associated to diapause are a common feature of facultatively sexual rotifers (Montero-Pau 2012; García-Roger and Ortells 2018; Chapter 3), which reproduce asexually and eventually sexually. These rotifers have a complex life cycle, in which the production of diapausing eggs is linked to sexual reproduction (see Chapter 2), which entails some costs. Firstly, because diapause implies an obligate dormant period (Schwartz and Hebert 1987; Hagiwara and Hino 1989; Marcus and Lutz 1998; see above), so delayed hatching implies longer generation times. Secondly, because since sex is required for the production of diapausing eggs –and it needs the participation of males–, females incur the so-called “twofold cost of sex” or “cost of males” due to the allocation

of resources to the production of males that do not contribute to the population growth (Maynard Smith 1978; Aparici et al. 2002; Serra and Snell 2009; Carmona et al. 2009; Stelzer 2011). Consequently, the switch from asexual to sexual reproduction, and the production of diapausing eggs in the rotifer life cycle is associated with costs derived from both diapause and sexual reproduction, which together decrease the population growth rate.

Replaying the tape of life

At the end of the twentieth century, Stephen Jay Gould in his book *Wonderful life* popularized the idea of a hypothetical experiment called “replaying life's tape” (Gould 1989). In this famous thought experiment, it would be somehow possible to “press the rewind button and (…) go back to any time and place in the past”– i.e., to rewind the history of life to any point back– and then “run the tape again and see if the repetition looks at all like the original” or is totally different. This idea is generally understood as a metaphor supporting Gould's philosophy of evolutionary contingency and has been associated to experimental evolution studies as a way of testing the reproducibility of evolutionary outcomes (Lobkovsky and Koonin 2012; Lang and Desai 2014; Orgogozo 2015; Fisher and Lang 2016).

Experimental evolution uses controlled laboratory conditions with defined selective pressures and has been recognized as a mighty approach in ecology and evolution to both detect short-term evolutionary responses (Kassen 2002; Garland and Rose 2009; Kawecki et al. 2012; Kang et al. 2016) and explore the genomic basis of adaptation (Barrett and Hoekstra 2011; Barrick and Lenski 2013; Matos et al. 2015).

Laboratory evolution experiments have a series of features that make them powerful tools (see Chapter 2). These experiments, in contrast to experiments in the field, allow to establish close replicates (Nakagawa and Parker 2015), to control experimental parameters, and to characterize the ancestral populations (Matos et al. 2015; Fisher and Lang 2016). However, despite the well-known advantages of experimental evolution, cautiousness is necessary when extrapolating laboratory results to nature, since the adaptation to specific laboratory conditions can lead to confounding effects or artifacts (Kawecki et al. 2012). Nevertheless, experimental evolution studies using laboratory populations can be used as a complement to studies with natural populations, providing a robust system to test hypotheses underlying evolutionary processes (Fisher and Lang 2016).

The revolution of the "omic" technologies

In last years, the fields of population genetics, molecular ecology, and conservation biology have been revolutionized by the advances in next-generation sequencing (NGS) techniques. NGS is a type of DNA sequencing based on massive sequencing techniques, yielding millions or billions of small reads of DNA. The molecular and technological advances together with the decreasing costs of "omic" technologies –e.g., genomics, transcriptomics, proteomics, and metabolomics– have produced large amounts of sequencing data (Mardis 2011; Esposito et al. 2016; Elmer 2016). Until recently, most knowledge in genomics was based on model organisms. However, the advances in NGS have allowed the utilization of non-model organisms in this type of studies (Van Straalen and Roelofs 2011; Stapley et al. 2010; Ekblom and Galindo 2011; Porcelli et al. 2015). Nevertheless, the genomic resources in non-model organisms are still scarce and limit the ability to uncover functionally relevant genes (Stinchcombe and Hoekstra 2008; Pavey et al. 2012; Alvarez et al. 2015).

Two of the most used "omic" technologies are genomics and transcriptomics. Firstly, genomics focuses on understanding the structure, function, and evolution of genomes. Through the characterization of the genomic variation, this technique allows to evaluate population structure and to identify genotypes that can be

responsible of adaptations (Elmer 2016; Oziolor et al. 2017). Secondly, transcriptomics is based on the study of gene expression at any given time in a cell in relation to some condition of interest (Van Straalen and Roelofs 2011). Comparative transcriptomic studies –i.e., comparing expression data– allow identifying differentially-expressed genes between populations or within a population when subject to different environments or experimental conditions (Eckblom and Galindo 2011; Da Fonseca et al. 2016).

Objectives and outline of the thesis

This thesis takes advantage of the above-mentioned technical advances and adopts the theoretical framework of evolutionary ecology in order to address the adaptive responses to unpredictable environments of rotifer populations using an experimental evolution approach.

The thesis is aimed at achieving the following goals:

(1) To study the adaptive response of life-history traits related to diapause in populations of the rotifer *Brachionus plicatilis* under two contrasting selective regimes (predictable vs unpredictable hydroperiod length) simulated in the laboratory.

(2) To elucidate the genomic basis of adaptation to environmental unpredictability of *B. plicatilis* populations evolved under the two selective regimes (predictable vs unpredictable), using genomic technologies.

(3) To explore the genetic expression in diapausing eggs produced by rotifer populations evolved under two fluctuating selective regimes (predictable vs unpredictable) and subjected to two diapause conditions (with and without an obligate period of diapause).

Taking into account these main objectives, the thesis –in the parts succeeding this general introduction (**Chapter 1**)– is organized as follows:

Chapter 2 describes the methodological context used in the development of the thesis. Firstly, it introduces the main biological features of the model species, the facultatively sexual rotifer *B. plicatilis*. Secondly, this chapter describes the experimental evolution design used in the development of the objectives of the thesis. In this chapter, the details on the establishment of the laboratory populations of *B. plicatilis* to be subjected to two fluctuating selective regimes (predictable vs unpredictable hydroperiod length) are provided, and their maintenance during the cycles of selection are specified. Finally, the molecular techniques used in the thesis are described: genotyping by

sequencing (GBS) and RNA sequencing (RNA-Seq). Therefore, Chapter 2 is an extension of the general introduction of the thesis, but it focuses on the methodology. Moreover, it provides some background to help the readers who are not familiar with the study organism and some methodological aspects developed in further chapters.

Chapter 3 studies two key life-history traits related to diapause that are key in the rotifer life cycle. This objective is addressed using an experimental evolution approach in laboratory populations of the rotifer *B. plicatilis* to evaluate the adaptive response to the two contrasting selective regimes (predictable vs unpredictable). According to theory, it is hypothesized the evolution of bet hedging as an adaptive response, in the laboratory populations under the unpredictable regime. Therefore, different life-history strategies (i.e., different combinations of the two traits studied) are expected to arise in each selective regime. In addition, in this chapter the time for divergence between both selective regimes is compared for the two diapause-related traits studied.

Chapter 4 analyses the genotyping-by-sequencing (GBS) data obtained from the populations at the evolution experiment. More specifically, data was obtained from rotifer clones founded from each laboratory population after evolving through seven cycles of selection in either of the two selective regimes (predictable vs

unpredictable). Additionally, GBS data from the origin population are used to evaluate the evolutionary trajectories during the evolution experiment. In this chapter, the genetic markers are related to the adaptation to unpredictable environments and associated to ecologically relevant phenotypic traits (those obtained in Chapter 3).

Chapter 5 performs a comparative analysis of transcriptome data using the diapausing eggs gathered from the studied laboratory populations after selection experiment (see Chapter 2). Genetic expression in diapausing eggs produced under both predictable and unpredictable selective regimes and subjected to two conditions of diapause is studied. A goal of this chapter is to obtain a catalogue of relevant genes for different moments in relation to entry into diapause, its maintenance and reactivation.

Chapter 6 discusses in general terms the main results obtained in this thesis, proposes prospective research, and sets out the most important conclusions.

2

The methodological context

Summary

This chapter provides an overview of the general methodology used in the development of the thesis objectives. Firstly, it describes the main biological features of the model species: the rotifer *Brachionus plicatilis*. Secondly, provides an overview of the usefulness and importance of experimental evolution approach as a tool in studies on evolutionary ecology. Thirdly, the experimental evolution design used throughout this thesis is described. The details on the establishment of *B. plicatilis* laboratory populations to be subjected to two contrasting selective regimes (predictable vs unpredictable) and their maintenance during eight cycles of selection are specified. Lastly, the main molecular techniques used throughout the thesis are described: genotyping by sequencing (GBS) and RNA sequencing (RNA-Seq).

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

Brachionus plicatilis (Müller 1786; Figure 2.1), belonging to the phylum Rotifera and the class Monogononta, has been recently proposed as a remarkable model organism to address population and evolutionary ecological studies (e.g., Fussmann 2011; Snell 2014; Declerck and Papakostas 2017; Stelzer 2017; Serra et al. in review), owing to the combination of biological features such as small size, high population growth rate and short generation time, and life cycle complexity that confer them unique methodological advantages (Kostopoulou et al. 2012; Declerck and Papakostas 2017; Serra et al. in review). In this chapter, these –and other– features that make monogonont rotifers especially suitable for experimental evolution studies are revisited (see below).

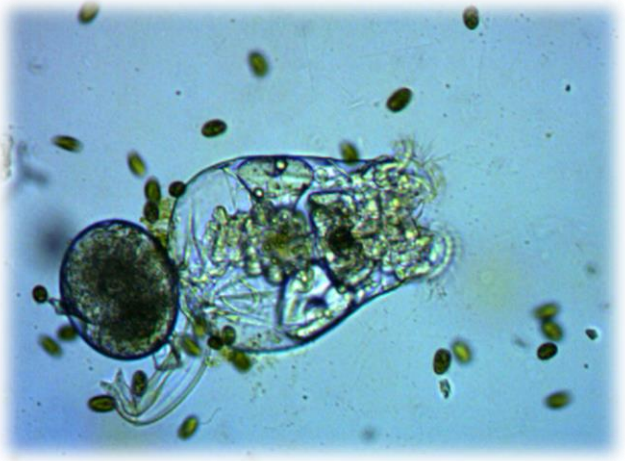


Figure 2.1. Microphotography of a *B. plicatilis* female carrying an asexual egg.

Rotifers are minute invertebrate metazoans –most of them measure between 100 and 500 µm (Hickman et al. 1997)– dwelling in a diverse array of aquatic and semiaquatic, macro- and microhabitats. The former are mostly freshwater, but also brackish and marine waterbodies, whereas the latter include the interstitial water in soils and the film of water on mosses and lichens of terrestrial habitats (Wallace et al. 2006; Segers 2008; Wallace and Smith 2009; Fontaneto and De Smet 2015). These microinvertebrates commonly occur in high densities and are important filter feeders on algae and bacteria, so that they constitute an important component of the microplankton community structure by transferring energy to higher trophic levels (Starkweather 1987; Walz 1997; Armengol et al. 2001).

Rotifers make up a phylum named Rotifera (i.e., wheel-animals; from latin *rota* meaning "wheel", and *ferre* meaning "to bear"), which is composed by more than 2,000 described species distinguished from other metazoans by the presence of a ciliated corona ("wheel organ") –used for locomotion and food gathering– and a muscular pharynx called mastax (Segers 2008; Wallace and Smith 2009; Wallace et al. 2015; Serra et al. 2018). The phylum has been classically divided into three large taxa, each of them typically being considered a class (although their rank varies among taxonomical schemes): Bdelloidea, Seisonidea, and Monogononta. Notwithstanding, to the date, the phylogenetic position of rotifers remains unresolved. There is molecular and morphological support for clustering together the traditional groups of Rotifera and the phylum Acanthocephala (Mark Welch and Meselson 2000; Sørensen and Giribet 2006; Segers 2008; Fontaneto and Jondelius 2011; Wey-Fabrizius et al. 2014) –with acanthocephalans being a sister group of Seisonidea (Sielaf et al. 2016)– within a larger clade, be it called Rotifera, Rotifera *sensu lato,* or Syndermata (e.g., Ahlrichs 1997; Dunn et al. 2014; Ruggiero et al. 2015). The four taxa differ in their morphology and ecology, but more peculiarly in their reproductive mode. Bdelloidea, including about 460 species, are obligate asexuals reproducing exclusively by parthenogenesis, and inhabit any wet or moist habitat (Fontaneto and Ricci 2004; Flot et al. 2013). Seisonidea, which includes only three described species,

are dioceous, obligate sexuals and live as epibionts on marine crustaceans (*Nebalia* species; Ricci et al. 1993). Acanthocephala, with about 1,150 species, have a highly modified morphology and are entirely sexual. Adult acanthocephalans are always intestinal parasites of vertebrates, engaging arthropods as intermediate hosts (Kennedy 2006; Goater et al. 2014). Finally, Monogononta, with about 1,600 described species, are cyclical parthenogens with facultative sexual reproduction. Most of them are planktonic, and despite freely living in fresh, brackish and marine waters, are well known as one of the major groups of zooplankton in continental waterbodies (Makarewicz and Likens 1979; Pace and Orcutt 1981; Segers 2008).

Cyclical parthenogenesis is therefore the defining feature of the life cycle in monogonont rotifers such as *Brachionus plicatilis*. In this life cycle (Figure 2.2), ameiotic parthenogenesis producing clonal females –asexual clonal proliferation– is combined with occasional bouts of male production and sexual reproduction (called mixis in the rotifer literature). The product of sexual reproduction is a dormant embryo, the so-called diapausing egg. Thereby, in these rotifers sex is linked to diapause.

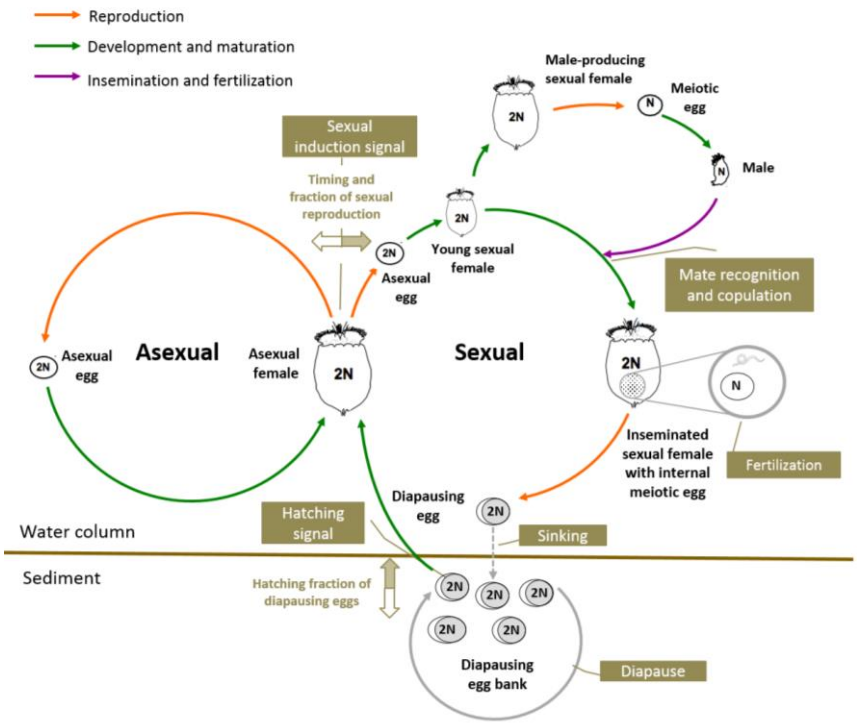


Figure 2.2. Life cycle of the rotifer *Brachionus plicatilis* (modified from Serra et al. in review), with emphasis on the reproductive, development and fertilization processes. Double arrows represent traits in which there is variation and are studied in this thesis.

In temperate climates, rotifer populations are not continuously active in the water column; rather, they are temporary and typically re-colonize the water column every year during the so-called planktonic growing season. The active population in the water column is re-initiated from diapausing egg hatchlings (Gilbert 1974; Pourriot and Snell 1983). These eggs (Figure 2.3) remain viable in the sediments of the ponds and lakes they inhabit forming part of

huge egg banks (Kotani et al. 2001; García-Roger et al. 2006). Hatchlings are asexual (amictic) females that parthenogenetically produce ameiotic subitaneous eggs, which hatch into daughters that are genetically identical to their mothers. This is a kind of clonal propagation that enables fast population growth and has the potential for the colonization of new habitats from only one or just a few founding females (Gómez et al. 2002). After this initial period of clonal propagation –during which population is composed by clonal lineages– sexual reproduction is induced by environmental cues that may differ among genera or species, such as photoperiod, population density, or diet (e.g., Gilbert 1974; Pourriot and Snell 1983; Schröder 2005). These cues trigger asexual females to produce sexual daughters as some fraction of their offspring, so sexual and asexual reproduction overlap. Sexual (mictic) females produce meiotic haploid eggs that develop either into males or, if fertilized, into diapausing eggs. Males are dwarf, functionally simple, and have shorter lifespan than females (King and Miracle 1980; Ricci and Melone 1998). The sexually-produced eggs undergo an embryonic diapause; they settle in the sediment and remain dormant for a period of variable length (Hagiwara and Hino 1989; Martínez-Ruiz and García-Roger 2015; Stelzer 2017).

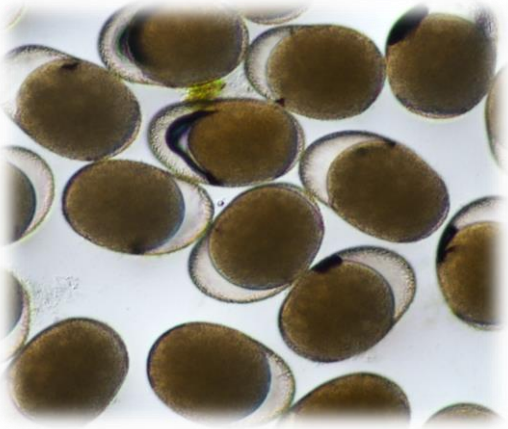


Figure 2.3. Diapausing eggs of the rotifer *Brachionus plicatilis.* Microphotography by Eduardo M. García-Roger.

Diapausing eggs survive adverse conditions allowing rotifer genotypes to endure unsuitable periods caused by abiotic (e.g., drought, extreme values of salinity and/or temperature, etc.) or biotic factors (e.g., competitors, predators, parasites, etc.). As mentioned above, these diapausing stages enable populations to recolonize local habitats when suitable conditions resume, as well as to disperse them among habitats (Moreno et al. 2016). Diapausing egg hatching takes place when suitable conditions resume in the water column and after receiving appropriate stimuli, so a new growing season begins and the genetic diversity stored in the egg banks is released. However, not all diapausing eggs hatch in the season following their production (Schröder 2005; Martínez-Ruiz and García-Roger 2015). The unhatched eggs often show prolonged diapause and accumulate in the sediment forming

diapausing egg banks awaiting for an adequate moment to hatch in what could be be a risk-spreading strategy (see Chapters 1 and 3). While in diapause, these eggs can remain viable for decades or even centuries (Marcus et al. 1994; Kotani et al. 2001; García-Roger et al. 2006). Diapausing egg banks are also reservoirs of the past genetic diversity and of the adaptive phenotypic variability in the population (Hairston 1996; Brendonck and De Meester 2003; Montero-Pau 2012), and are essential for the long-term persistence of rotifer populations (García-Roger et al. 2006). As a whole, this life cycle is considered an adaptation to temporally varying environments (Serra and King 1999).

The switch between the two reproductive modes –asexual and sexual– is density dependent in the genus *Brachionus*. Sex is induced by an infochemical produced by the females and released into the medium (Gilbert 1963; Carmona et al. 1993; Stelzer and Snell 2003; 2006). This infochemical is a protein called mixis-inducing protein (Snell et al. 2006), whose concentration increases with rotifer population density and triggers sexual reproduction when it reaches a density threshold (Snell and Boyer 1988; Carmona et al. 1993; 1995). Interestingly, this mechanism is analogous to quorum sensing in bacteria (Miller and Bassler 2001; Kubanek and Snell 2008). Indeed, rotifers are among the few examples in metazoans exhibiting such behaviour (Kubaneck and Snell 2008).

Among planktonic rotifers in the class Monogononta, the best-known taxon is the *B. plicatilis* species complex. This is a complex of cryptic species (i.e., having great morphological similarity; Gómez et al. 2002; Ortells et al. 2003) that includes at least fifteen species (Gómez 2005; Suatoni et al. 2006; Mills et al. 2017). Classical, morphologically based taxonomy considered these species to be a single species called *B. plicatilis*. However, the development of molecular taxonomy has made possible to unmask these cryptic species (Fontaneto et al. 2007; Montero-Pau et al. 2011). In this thesis, the species *B. plicatilis sensu stricto* (Müller 1786) belonging to this cryptic species complex was used as model organism.

Evolution in experimental populations

Experimental evolution is the study of evolutionary processes occurring in laboratory and field populations in response to conditions imposed by the experimenter (Kawecki et al. 2012). It has been recognized as an alternative research approach that offers the opportunity to study evolutionary processes experimentally in real time, i.e., evolution in action (Kawecki et al. 2012). In the last decades, many studies have valued experimental evolution as a powerful and versatile tool in ecology and evolution for (1) detecting short-term evolutionary responses, and (2) understanding the mechanisms underlying patterns of genetic and

phenotypic diversity in populations evolved under controlled conditions during many generations (Kassen 2002; Buckling et al. 2009; Garland and Rose 2009; Kawecki et al. 2012; Teotonio et al. 2017). In addition to the benefit of establishing lab-controlled selective pressures for tracking evolutionary trajectories, two other advantages of experimental evolution approaches are replication (i.e., several replicates can be performed in each selective regime; Elena and Lenski 2003; Garland and Rose 2009; Matos et al. 2015; Schlötterer et al. 2015) and reproducibility (Lang and Desai 2014; Fisher and Lang 2016).

Laboratory experimental evolution has the potential to evidence adaptation to several selective pressures occurring over a relatively reduced number of generations –although this number depends on the studied organism– and has been widely applied to a phylogenetically-broad range of organisms. Since short generation time and tractability in laboratory settings are key features of organisms used in experimental evolution studies, the majority of such studies have focused on viruses and bacteria (e.g., Lenski et al. 1991; Elena and Lenski, 2003; Ackermann et al. 2007; Beaumont et al. 2009; Cooper and Lenski 2010). However, many other features over which it may be interesting to study the evolution in action are unique to eukaryotes (e.g., multicellularity, the extent of sexual reproduction, etc.). Consequently, the studied organisms have been expanded to include fungi (e.g., Fisher and Lang 2016), plants

(e.g., Gervasi and Schiesti 2017), fruit flies (e.g., Burke and Rose 2009), nematodes (e.g., Teotónio et al. 2017), among many others.

Among metazoans, several studies have used experimental evolution approaches to address adaptation to environmental fluctuation. For instance, recent studies in *Drosophila* have demonstrated the adaptation to temperature variation (Kellerman et al. 2015; Adrián et al. 2016), and to both the unpredictable and unpredictable variation between stressful and benign conditions (Kubrak et al. 2017). Another instance of evolution studies on variable environments with metazoans are laboratory experiments performed with the nematode *Caenorhabditis elegans* (Teotónio et al. 2017). One of these studies tested the evolution of life-history traits in response to different polluted environments by using selective regimes in which organisms faced fixed levels of either of the pollutants, and regimes in which the two pollutants were alternated (Dutilleul et al. 2014). Results showed quick evolutionary responses to anthropogenically induced selection pressures. Another study evaluated the importance of maternal effects in adaptation to fluctuating environments by exposing *C. elegans* populations to normoxia–anoxia larval hatching environments, with either regular or irregular fluctuations (Dey et al. 2016). Experimental evolution results showed the evolution of anticipatory maternal effects. Remarkably, the above-mentioned studies have focused on two well-established model organisms. At

this sense, it is worthy of note that monogonont rotifers have been recently recognized as suitable model organisms for experimental evolution (Fussmann et al. 2003; Declerck et al. 2015; Declerck and Papakostas 2017).

Monogonont rotifers have several features that make them an excellent model for experimental evolution studies (Table 2.1). Firstly, their small size (usually < 1 mm) and short generation time allow to maintain large laboratory populations in small volumes, achieving high population densities. Large population sizes are essential to study evolution in laboratory populations in order to reduce the effect of random genetic drift (Jónás et al. 2016; Declerck and Papakostas 2017). Moreover, rotifers have high rates of population growth and short generation times (Bennett and Boraas 1989), what allows the study of rapid trait evolutionary responses to selective regimes (Fussmann 2011; Declerck and Papakostas 2017; Walczyńska et al. 2017) and to obtain comprehensive time series of data over many generations within an affordable experimental time. Secondly, monogonont rotifers are euryphagous, generalist filter feeders –feeding on bacteria, algae, protozoa, and yeast, as well as organic detritus (Wallace et al. 2015)– what makes them easy to culture and maintain in the laboratory. Additionally, monogonont rotifers are the only aquatic metazoans that have been found to be able to grow under steady-state conditions in chemostats –i.e., continuous culture– (Walz

1993). Besides improving culture and maintenance under controlled laboratory environments in automated systems, the achievement of a steady state also reduces the interference of genetic drift with selection processes (Declerck and Papakostas 2017). Thirdly, the cyclical parthenogenetic life cycle of monogonont rotifers –combining both asexual and sexual reproduction– benefits of both rapid clonal proliferation and genetic recombination by sexual reproduction. Sex generates new genetic variants in a short time span, which allows selection to act (Becks and Agrawal 2012; Declerck et al. 2015). Due to asexual reproduction, rotifers can be easily cloned in the laboratory, which permits to obtain huge numbers of isogenic individuals. Establishment of clones allows for replication of genotypes in experiments and to control for genetic variation (Declerck and Papakostas 2017; Serra et al. in review). Additionally, diapausing eggs produced as the result of sexual reproduction permit the long-term maintenance of stocks in the laboratory and ease the foundation of clonal lines to be used in experiments. Finally, a number of studies have shown that evolutionary and ecological time scales can overlap in rotifers (Yoshida et al. 2003; Fusmann et al. 2003). Such overlap creates a potential for the study of eco-evolutionary dynamics in these metazoans (Fussmann et al. 2007; Schoener 2011).

Table 2.1. Features of cyclically parthenogenetic rotifers that make them suitable model organisms for experimental evolution studies (after Serra et al. in review).

Features	Methodological advantage
Small size	Large laboratory populations can be maintained in small volumes.
Short generation time and high growth rate	(1) Rapid evolutionary responses; (2) Ease of data collection over many generations; (3) Moderate experimental times.
Euryphagous	Maintenance in simple and inexpensive culture media.
Ecological adaptability	Ease of culturing and maintenance under controlled laboratory environments in automated culture systems.
Complex life cycle	Asexual and sexual reproduction in the same genetic background.
Clonal proliferation	(1) Establishment of isogenic lines, (2) genotype replication, and (3) control of genetic variation in experiments.
Sexual reproduction	Generation of genetic variants, which allows selection to act.
Production of diapausing eggs	(1) Easy establishment of clonal lines, and (2) long-term maintenance of stocks in laboratory.
Environmental sex induction	Control of sexual reproduction under experimental conditions.
Haploid males	Development of inbred lines in the laboratory.

Experimental evolution approaches have been successfully performed in monogonont rotifers for a variety of selective factors (see Table 2.2), both abiotic (e.g., temperature, Walczynska et al. 2017; salinity, Scheuerl and Stelzer 2013; and hydroperiod, Smith and Snell 2012) and biotic (e.g., predator-prey dynamics, Fussmann 2003; Haafke et al. 2016; food and nutrient limitation, Becks and Agrawal 2010; Scheuerl and Stelzer 2013; Declerck et al. 2015). For instance, Becks and Agrawal (2010) tested the evolution of the rate of sexual reproduction (i.e., the fraction of sexual daughters; see Figure 2.2) in *Brachionus calyciflorus* populations subjected to homogenous vs heterogeneous environments. Homogeneous environments were characterized by either high or low food quality –according to its nitrogen concentration–, while heterogeneous environments were mimicked through the sequential transfer of rotifers (i.e., migration) between the two types of homogeneous environments. Experimental *B. calyciflorus* populations growing in the heterogeneous environment evolved higher rates of sexual reproduction than those growing in the two homogeneous environments, in which the rate of sex rapidly evolved towards zero. A subsequent research by Becks and Agrawal (2012) focused on the adaptation of *B. calyciflorus* populations to fixed vs novel environments. In that study, experimental populations –previously adapted to two different food conditions (see Becks and Agrawal, 2010)– were either subjected to the same regime of environmental

conditions or exposed to a novel regime, by changing from low to high food quality, and viceversa. Results showed that populations subjected to novel environments evolved higher rates of sex. In another study, Smith and Snell (2012) used *B. plicatilis* populations to test the evolution of (1) the timing of sex, (2) the rate of sexual reproduction, and (3) the production diapausing eggs under different hydroperiod regimes (permanent vs ephemeral). Experimental populations showed earlier timing of sex, higher rates of sexual reproduction and higher diapausing egg production under the ephemeral selective regime than in the permanent one. Recently, Declerck et al. (2015) performed an experimental evolution study in which field-derived *B. calyciflorus* populations were allowed to evolve under two different regimes characterized by their stoichiometric food quality (i.e., phosphorous-limited vs phosphorous-repleted microalgae). Adaptation of rotifer populations in biomass, mortality rates and body stoichiometry to nutrient limitation in microalgae was tested in a subsequent common-garden experiment where populations were exposed to the two different food quality treatments after the selection experiment. Results showed that rotifer populations previously evolved in P-limited food regime suffered lower mortality and reached twice the steady state biomass when exposed to a P-limited food environment than the populations previously evolved under the P-repleted regime.

Overall, the above-mentioned studies showed rapid evolution of rotifer populations (≤ 125 days; Declerck and Papakostas 2017). They also evidence experimental evolution in rotifers as an emergent field of research. However, the number of studies is still reduced. Interestingly, many of them have focused on sex-related life-history traits. For instance, two of the traits studied are the propensity for sexual reproduction (i.e., timing of sex; Becks and Agrawal 2010; 2012; Smith and Snell 2012; Haafke et al. 2016) and diapausing egg production (Smith and Snell 2012). Moreover, these pioneer studies practiced the exposure of evolving populations to changing conditions, either by fluctuating environmental conditions (Smith and Snell 2012) or by exposing the evolving populations to sudden novel environments (Becks and Agrawal 2012; Declerck et al. 2015)–. This supports the suitability of the experimental evolution approach to test evolutionary hypotheses in rotifer diapause-related traits when exposed to different degrees of environmental unpredictability.

Table 2.2. Experimental evolution studies using monogonont rotifers as model organism (updated from Declerck and Papakostas 2017). NR: not relevant

Selective factor	Life-history traits assayed	Rotifer species	Reference
NR	Propensity for sex	*B. calyciflorus*	Fussmann et al. 2003
Spatial heterogeneity	Propensity for sex Fecundity	*B. calyciflorus*	Becks and Agrawal 2010
Temporal heterogeneity	Propensity for sex Fecundity	*B. calyciflorus*	Becks and Agrawal 2012
Habitat permanence	Propensity for sex Diapausing egg production Life span Fecundity	*B. plicatilis*	Smith and Snell 2012
Salinity and food quantity	Population growth rate	*B. calyciflorus*	Scheuerl and Stelzer 2013
Nutrient limitation	Biomass Mortality rate	*B. calyciflorus*	Declerck et al. 2015
Temperature	Body size Asexual egg size	*B. plicatilis*	Walczynska et al. 2017
Prey type fluctuation	Propensity for sex	*B. calyciflorus*	Haafke et al. 2016

The experimental evolution design

Origin populations

Laboratory populations of *B. plicatilis* used in this thesis were founded from natural populations inhabiting nine Spanish Mediterranean saline ponds and lakes (Table A1; Franch-Gras 2017). Due to particular climate, orography and hydrogeology in the Mediterranean region, water bodies therein are characterized by having a high degree of seasonality and uncertainty at various temporal scales (Blondel et al. 2010). These nine saline ponds and lakes in which *B. plicatilis* was found (Franch-Gras et al. 2017b) are located in eastern Iberian Peninsula, in an area of approximately 240 km^2. The climate in this area is semiarid, with an average annual rainfall of ca. 343 mm and a mean temperature of around 14 °C (Franch-Gras 2017). This set of ponds and lakes includes water bodies with a wide range of sizes (water-surface area from 0.013 to 119 ha.), characterized by being shallow (maximum depth ca. 1m), non-permanent, and brackish or saline (salinity ranging from 1.8 to 54 g L^{-1}). The main water inflow in these waterbodies is through rainfall, but some of them are also connected to groundwater (Gómez-Alday et al. 2014). There is variability in the flooding pattern among these nine ponds and lakes according to the characterization from satellite images recently performed by Franch-Gras et al. (2017a). By means of satellite data, these authors

showed that this set of water bodies covers a wide range of predictability in their flooding patterns (from highly unpredictable to almost completely predictable). This variation in environmental predictability is relevant from the point of view of rotifers (Franch-Gras et al. 2017a), the organism on focus in this thesis.

A total of 30 *B. plicatilis* clones were founded from each of the nine saline ponds and lakes described above. Diapausing eggs were isolated from the sediment using a sugar flotation technique (Gómez and Carvalho 2000). Those diapausing eggs that looked healthy were transferred individually into 96-multiwell plates (Nunc^{TM}) and induced to hatch under standard hatching conditions (25 °C, 6 g L^{-1} salinity and constant light; García-Roger et al. 2005; 2006). Hatchlings were monitored daily and clonal lines were established by asexual proliferation of the resulting neonate females. Since *B. plicatilis* belongs to a cryptic species complex, clonal lines were identified to the species level by genetic analysis of cytochrome c oxidase subunit I (COI) based on PCR-RFLP (Campillo et al. 2005). After identification, clonal lines from each field population were maintained in 15 mL stock cultures at 12 g L^{-1} salinity and 20 °C until used in the experiment.

Laboratory experimental populations: establishment and maintenance

Six, genetically diverse, laboratory populations were established for being subject to the experimental evolution study. In order to generate these laboratory populations, three ovigerous asexual females from each of the 30 clonal lines per field population were placed together in each of six 950-mL glass flasks (each containing 810 females per laboratory population). Thus, the initial genetic composition was the same for each one of the six experimental laboratory populations. By creating these multiclonal populations (from clones founded from diapausing eggs collected in the field), it was intended to obtain experimental populations with high genetic variability integrating the levels of genetic variation of the nine, natural rotifer populations.

The experimental rotifer populations were grown in chemostat cultures (Figures 2.4 and 2.5). These are continuous flow cultures in which fresh medium is constantly added at the same defined rate that culture is removed (Gresham and Duham 2014). One of the most important features of chemostats is that organisms can be grown in a physiological steady state under constant environmental conditions. In this steady state, growth occurs at a specific, constant rate, and physicochemical parameters in the culture –e.g., temperature, pH, gas concentration– are controlled and kept

constant during the experiment (Walz 1993; Pir et al. 2012; Gresham and Duham 2014). Rotifers are so far the only metazoans reported to be capable of reaching steady state population growth in continuous cultures (Walz 1993). This unique feature was exploited in the experimental design.

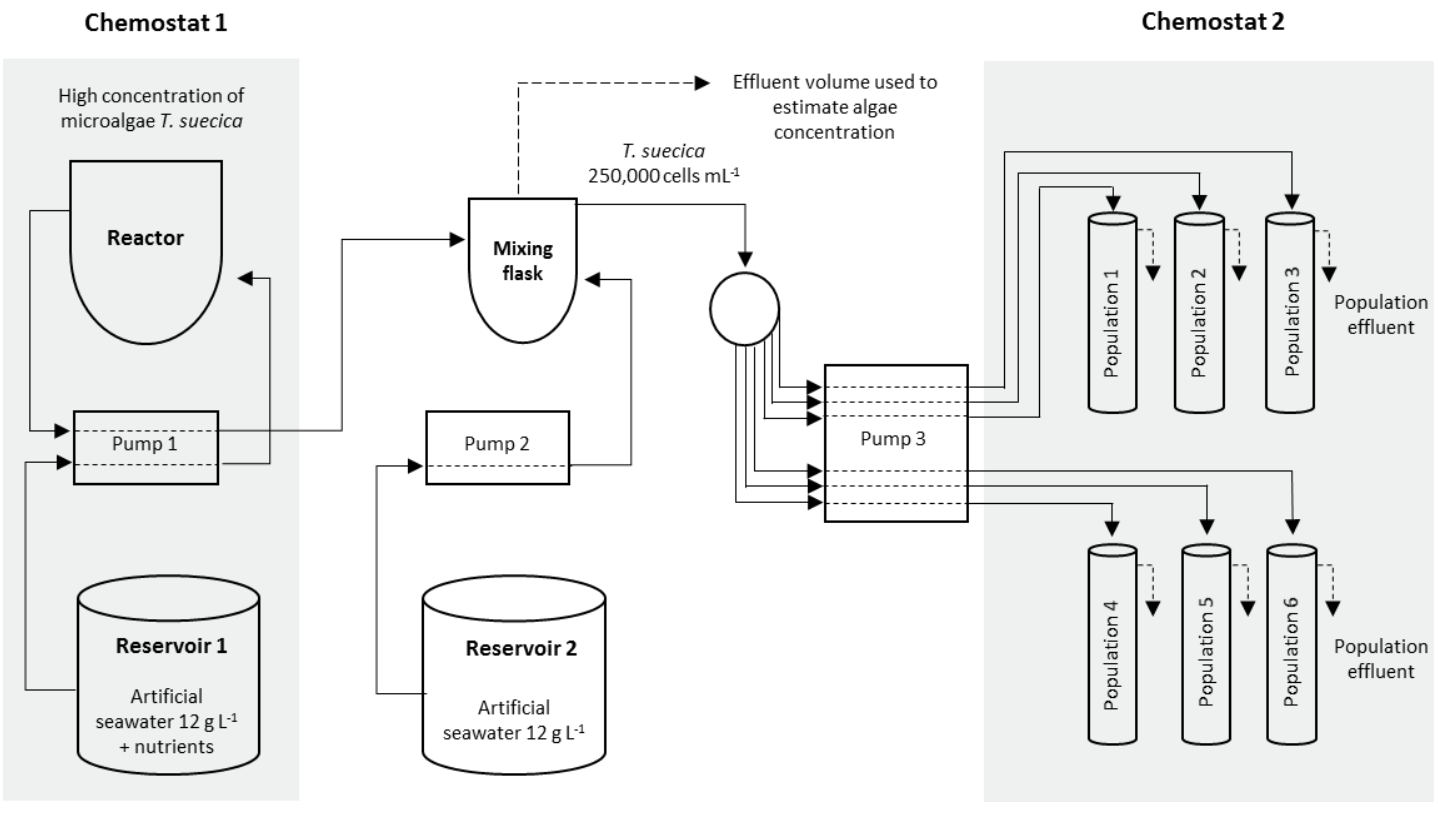
This design involved not only the experimental rotifer populations but also microalgae auxiliary cultures (see details below), which were grown using a two-stage chemostat system (Figure 2.4). A two-stage chemostat system consists of two separate chemostats. The first one is employed for the continuous growth of the microalgae to be used as culture medium of the rotifer populations. In this first chemostat, the dilution rate is keep constant. This allows for the maintenance of microalgae growing exponentially under constant physicochemical conditions (Pir et al. 2012). This system minimizes fluctuations in food supply and provides constant, high-quality microalgae to the experimental rotifer population cultures. These populations are cultured in the second chemostat, to which the culture medium flows at a fixed rate from the first chemostat. In between these two chemostats, a mixing flask is placed to check and adjust (if necessary) the concentration of the microalgae supplied (Walz 1993).

In the experimental design, the inflow rotifer culture medium consisted of the flagellate microalgae *Tetraselmis suecica* (250,000

cells mL^{-1}) continuously cultured in f/2 medium (Guillard and Ryther 1962) made with artificial seawater (Instant Ocean®, Aquarium Systems) at 12 g L^{-1} salinity. This microalgae was cultured at high concentration in a 2-L reactor (Chemostat 1; Figure 2.4) with a dilution rate of 0.410 mL min^{-1} using a peristaltic pump (Pump 1; Figure 2.4). Fresh medium –i.e., artificial seawater at 12 g L^{-1} salinity plus nutrients was supplied from a reservoir (Reservoir 1; Figure 2.4) to the microalgae culture at the constant flow rate of 0.410 mL min^{-1}, and removed from the reactor at the same rate, thus maintaining the culture volume constant. The culture medium flows from the reactor to a mixing flask in which microalgae concentration was estimated daily using an automatic cell counter (Celeromics Technologies S.L.). If necessary, a second pump (Pump 2; Figure 2.4) supplying artificial seawater at 12 g L^{-1} salinity was used to adjust the desired microalgae concentration in the mixing flask (250,000 cells mL^{-1}; Figure 2.4) before being supplied to the second chemostat. This flask was wrapped with aluminum foil to avoid algae growth. The second chemostat was, in fact, a device consisting on several glass flasks (Population 1-6 flasks; Figure 2.4) containing 950 mL of culture medium in which the experimental rotifer populations were allowed to grow. These flasks were covered with black, plastic bags to block light incidence. Experimental rotifer populations were maintained with a dilution rate of 0.15 day^{-1} using a third pump (Pump 3; Figure 2.4).

Chemostat's effluent volume from each experimental population was filtered out to retain diapausing eggs (see below). All chemostat components were connected with silicone tubes of 0.89 mm of diameter. In order to maintain culture homogeneity, the reactor, as well as the mixing and the population flasks, were aerated by means of air pumps. Air was sterilized through a 0.2 µm filter (PTFE-Filter; Sartorius Midisart) to avoid contamination. All the experiments were conducted in a walk-in growth chamber at 20 °C and 12L:12D photoperiod at standard light intensity (≈35 µmol quanta m^{-2} s^{-1}).

Figure 2.4. Two-stage chemostat system scheme (modified from T. Micó, personal communication). Reactor is the chemostat 1 and population flasks 1-6 correspond to the chemostat 2. Reservoirs are used for the inflow of artificial seawater (with and without nutrients) in order to maintain the desired concentration of microalgae. The mixing flask between both chemostats allows for the adjustment of microalgae concentration to be supplied to the rotifer experimental populations. Arrows represent silicone tubes that connect the different flasks and pumps.


Chemostat 1
Chemostat 2
High concentration of microalgae T. suecica
Reactor
Pump 1
Reservoir 1
Artificial seawater 12 g L-1 + nutrients
Mixing flask
Effluent volume used to estimate algae concentration
T. suecica 250,000 cells mL-1
Pump 2
Reservoir 2
Artificial seawater 12 g L-1
Pump 3
Population 1
Population 2
Population 3
Population effluent
Population 4
Population 5
Population 6
Population effluent

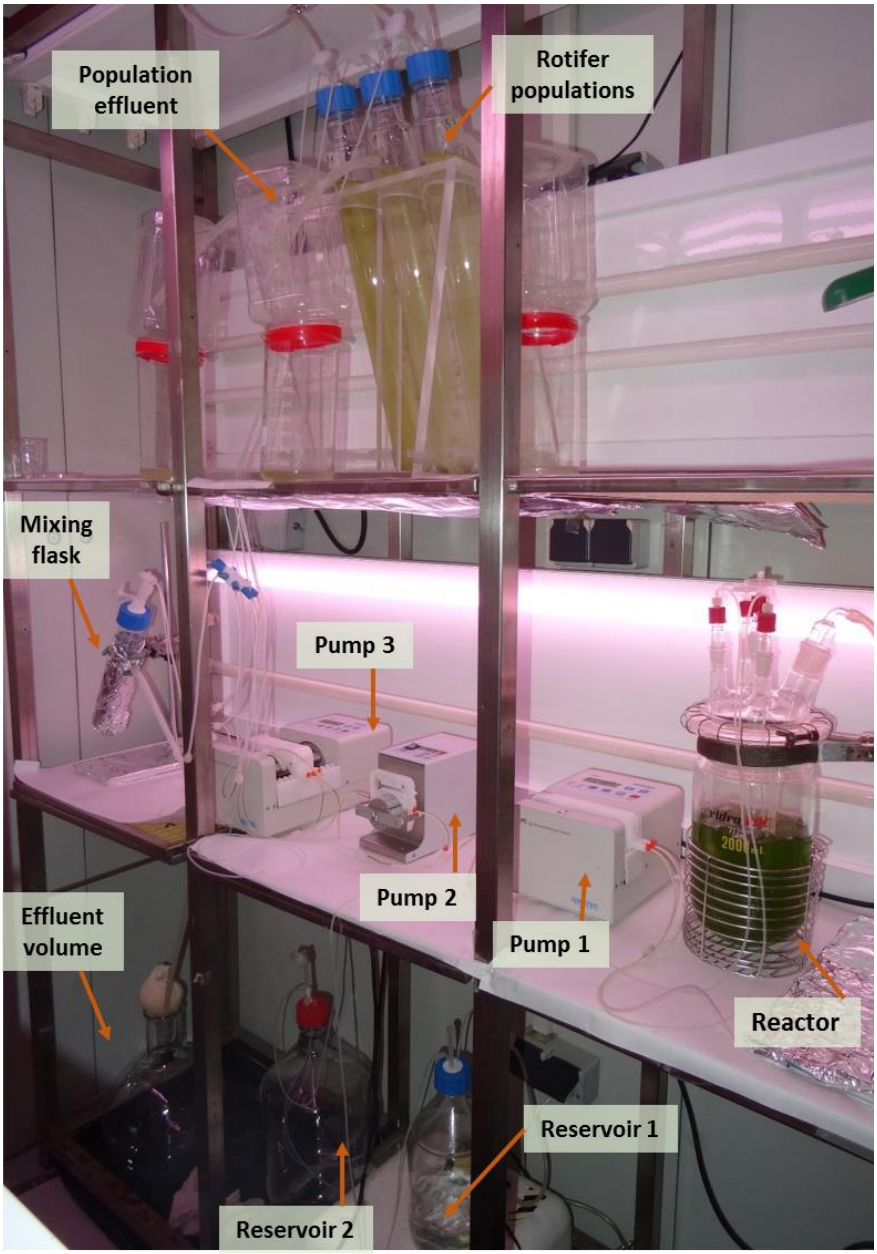


Figure 2.5. Chemostat set-up for experiments on rotifer adaptation to environmental unpredictability.

Selective regimes

From the six experimental populations, three of them (working as replicates) were randomly assigned to each of the two experimental environmental regimes under which they independently evolved through a series of eight growing cycles (Figure 2.6). Both experimental regimes fluctuated in hydroperiod length, but in one of them, the length of the growing cycles was kept constant, whereas it varied in the other. Thus, it is assumed that these regimes were predictable and unpredictable, respectively, by the rotifers. Notice, however, that to keep the experimental design logistically affordable while sufficiently powerful to detect differences between regimes, the fluctuation pattern (i.e., the particular sequence of different growing cycle lengths) was the same for the three unpredictable replicate populations. By doing so, the experimental design tests for the effect of this particular fluctuation pattern and not for any unpredictable fluctuation in growing-cycle length. The end of each growing season, which in nature can be caused by biotic or abiotic stresses, was simulated experimentally by filtering the cultures. For this experimental simulation, the successive growing cycles were initiated with the hatchlings of diapausing eggs produced in the previous growing cycle.

The length of each growing cycle in the predictable regime was 28 days. Field observations have shown that growing seasons of this length and even shorter are not uncommon (Franch-Gras et al. 2017a). Due to the short generation time of *Brachionus* asexual females (4.6 days in the experimental conditions used here; Gabaldón and Carmona 2015), this period was long enough to reach population densities for initiating sexual reproduction and to produce diapausing eggs (e.g., Gabaldón et al. 2015). Sexual females were observed on the 5th day of experimental growing cycles (results not shown), and diapausing eggs typically appear 2-3 days after fertilization (Tortajada et al. 2009). Because the obligate period of diapause can be as short as 3-4 days (Martínez-Ruiz and García-Roger 2015), this means that there can be a minimum of ca. 10 days from the hatching of a single diapausing egg to the production of a new sexual generation.

The length of the growing cycles fluctuated in the unpredictable regime, but averaged 28 days. The length of each growing cycle was randomly assigned using a custom R script (R Core Team 2015) following these steps: (1) 1000 rows of seven integers with uniform distribution in the range 4-53 were simulated; (2) the sequences averaging less than 27 or more than 29 were discarded; (3) the sequence with the highest variance was used. In this way, the fluctuating pattern was not prejudiced, but one with high fluctuation and an average growing-cycle length similar to that in

the predictable regime was obtained (Figure 2.6). All treatments were run synchronically, involving a total of 448 days (growing cycles plus diapause periods).

During each growing cycle, the effluent volume from each experimental rotifer culture was filtered through a 30-µm Nytal mesh sieve and recovered in a container. This mesh size ensured that individuals and eggs were retained. Every 4 days, the material retained on the filters was transferred to Petri dishes with saline water, and the diapausing eggs obtained were counted. These eggs were then dehydrated and kept at 4°C in darkness until the start of the next growing cycle. At the end of each growing cycle, rotifer population cultures were also filtered using the same type of mesh sieve that for the effluent. Diapausing eggs were collected and counted under a stereomicroscope. Afterwards, these diapausing eggs were also dried out at 25 °C and stored in the darkness at 4 °C for 28 days. To estimate diapausing egg production during a growing cycle, both these counts plus those of diapausing eggs collected from the population's effluent volume during the growing cycle were taken into account. After the dormancy period (28 days), 1,000 diapausing eggs per population (except for the shortest cycle) were induced to hatch under constant light (García-Roger et al. 2006), 12 g L^{-1} salinity and 25 °C in Petri dishes during four days. All newborn females and unhatched diapausing eggs were used to re-establish each population for the following growing cycle. As a

consequence of introducing unhatched eggs, recruitment from the egg bank might occur at any time during the growing cycle (i.e., delayed hatching can take place) or might even contribute to subsequent growing seasons, mimicking what may happen in nature.

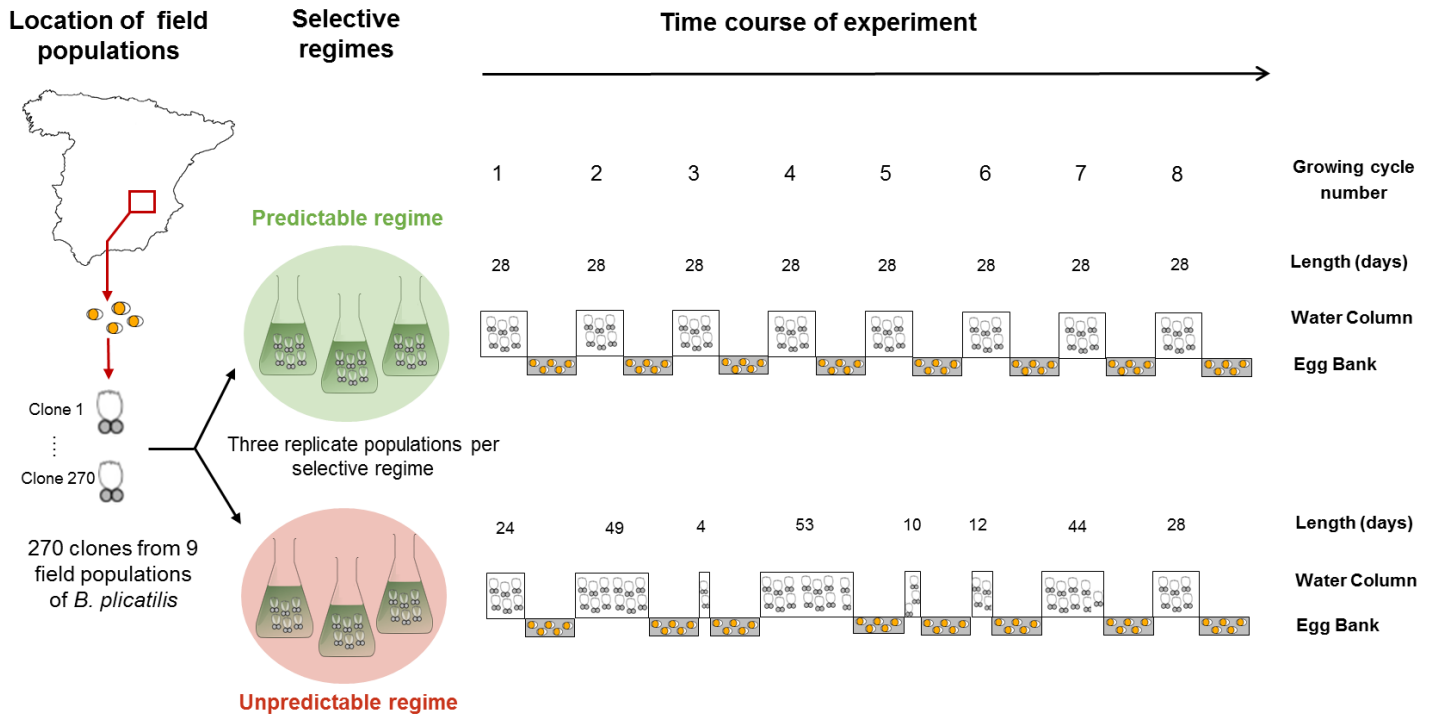


Figure 2.6. Schematic diagram of the experimental evolution design. Growing cycles of the active population in the "water column" and diapause in the "egg bank" are displayed. The duration of the unsuitable period was 28 days in all cases.

Molecular techniques

In achieving the thesis objectives, two main molecular techniques were used: (1) genotyping by sequencing (GBS), in Chapter 4; and (2) RNA Sequencing (RNA-seq), in Chapter 5. Here, the generalities of these molecular procedures are outlined with the intention of providing a better background to the readers not familiar with them (see Box 2.1 for more details). Furthermore, relevant, specific details of these methodologies are detailed in the corresponding chapters 4 and 5.

Genotyping by sequencing (GBS)

Identifying genetic signatures of selection at the genome level in wild and/or experimental populations is key to elucidate the genetic basis of ecological adaptation. The new technologies of high throughput next-generation sequencing (NGS) that have been rapidly developed during the last decade, no longer restrict the genomic studies to model organisms, but can be applied to almost any organism (see Chapter 1), even in species with scarce genomic resources such as the rotifer *B. plicatilis*. These techniques allow identifying those genes that underlie the adaptations in life-history traits and therefore the integration of genetic and ecological data (e.g., Hudson 2008; Stapley et al. 2010). One of the approaches recently developed by using NGS is genotyping by sequencing

(GBS), which has been applied successfully in a wide range of species (e.g., plants, metazoans, etc.). This sequencing approach has been described as simple but robust, and relies on the usage of restriction enzymes to reduce genome complexity, thus capturing only samples of the whole genome between restriction sites (Elshire et al. 2011). The GBS approach allows to identify and genotype thousands of single nucleotide polymorphisms (SNPs) in individuals from different populations (Davey et al. 2011; Narum et al. 2013). These SNPs are molecular markers, which permit to identify putatively genome locations with signatures of selection (Stapley et al. 2010).

The GBS method is outlined in Figure 2.7. Basically, after genomic DNA extraction (Step 1; Figure 2.7), DNA library is constructed. For this purpose, each genomic DNA sample is digested (Step 2; Figure 2.7) by a restriction enzyme (e.g., *ApeK1*; see Chapter 4). This fragmentation is sequence-dependent, and only those DNA fragments preceded by a cutting sequence of the restriction enzyme are sequenced. Digested DNA fragments are then complemented by adapters, and a specific barcode is added for each sample (Step 3; Figure 2.7). Then, specific DNA fragments – those with adapters and barcode– from all samples are pooled (Step 4; Figure 2.7). Due to pooling, the barcode is essential for samples to be distinguished and sorted in further data analyses. Pooled DNA is amplified by PCR (Step 5; Figure 2.7) and sequenced

on a NGS platform yielding single-end reads (Step 6; Figure 2.7). The raw sequence data obtained is further analyzed using bioinformatics tools (see Chapter 4).

In this thesis, the extraction of genomic DNA was performed in clones founded from diapausing eggs collected in the seventh cycle of selection from the evolution experiment. In total, DNA was extracted from 180 samples corresponding to 30 clones of *B. plicatilis* per six experimental populations –three replicates per selective regime– (see Figure 2.7 and Chapter 4). In order to obtain the optimal quantity of DNA from each sample for sequencing, each rotifer clone was mass-cultured in 1.5 L during 10 days at 25 °C. On the tenth day, when there were about 16,000 individuals per rotifer clone, DNA extraction was performed (see Chapter 4 for details).

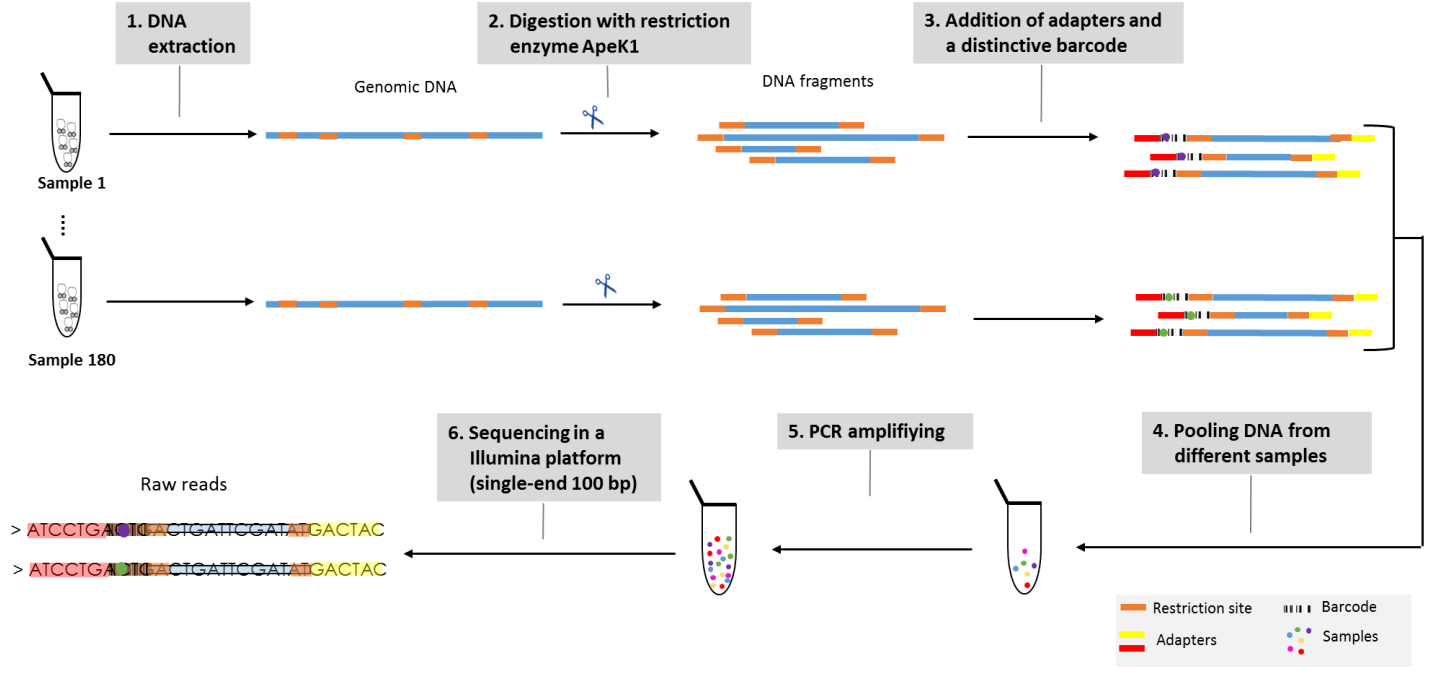


Figure 2.7. Scheme of the GBS protocol followed for the 180 *Brachionus plicatilis* samples obtained from populations evolving under two selective regimes of environmental predictability (for details see the text in this chapter and Chapter 4).

RNA Sequencing (RNA-Seq)

Recent advances in NGS have also revolutionized the field of transcriptomics, which is the study of the complete set of RNA transcripts that are produced by the genome under specific circumstances. RNA Sequencing (RNA-seq) technique has rapidly become an important tool in ecological and evolutionary research (Todd et al. 2016). This technique is based on the sequencing of complementary DNA (cDNA) through reverse transcription of mRNA (Wang et al. 2009). RNA-seq data allow the study of differences in gene expression patterns between populations and/or individuals within populations with different phenotypes or under different environmental conditions (Ekblom and Galindo 2011; Da Fonseca et al. 2016).

The general procedure for RNA-seq protocol is described in Figure 2.8. Briefly, after total RNA extraction (Step 1; Figure 2.8), an RNA library is constructed for each biological sample. In order to build up an RNA library, the enrichment of mRNA with a standard poly-A based method is necessary (Step 2; Figure 2.8). This step is used to remove non-mRNA from the samples. After it, mRNA is chemically fragmented (Step 3; Figure 2.8). This is a kind of random fragmentation, sequence-independent and is generally considered to produce uniformly distributed fragments of mRNA (Wang et al. 2009; Griebel et al. 2012). Then, complementary DNA (cDNA) is

synthetized by reverse transcription (Step 4; Figure 2.8). The cDNA is produced from the mRNA of a specific tissue or life stage and used to be sequenced. Once cDNA is obtained, Illumina adapters and a specific barcode for each sample are added to each cDNA fragment by means of PCR (Step 5; Figure 2.8). Afterwards, cDNA from all samples is pooled (Step 6; Figure 2.8) in order to be amplified, and sequenced on a NGS-platform yielding single-end reads (Steps 7 and 8; Figure 2.8). Once cDNA is sequenced, the raw sequence data are analyzed using bioinformatics tools (see Chapter 5).

In this thesis, total RNA extraction was performed in diapausing eggs harvested from each evolved laboratory population in the last cycle of selection (eighth growing cycle) of the evolution experiment (Figure 2.8; see Chapter 5 for more details). The collected diapausing eggs per laboratory population were divided into two treatment groups to be subjected to different diapause conditions. Note that the number of eggs was the same in each sample (5,000 diapausing eggs). Therefore, a total of 12 samples were obtained from three laboratory replicate populations per two selective regimes (predictable vs unpredictable) per two diapause conditions (See Chapter 5 for details). Once RNA was obtained and the RNA quantity and quality were assessed, RNA-Seq was performed following the protocol detailed above.

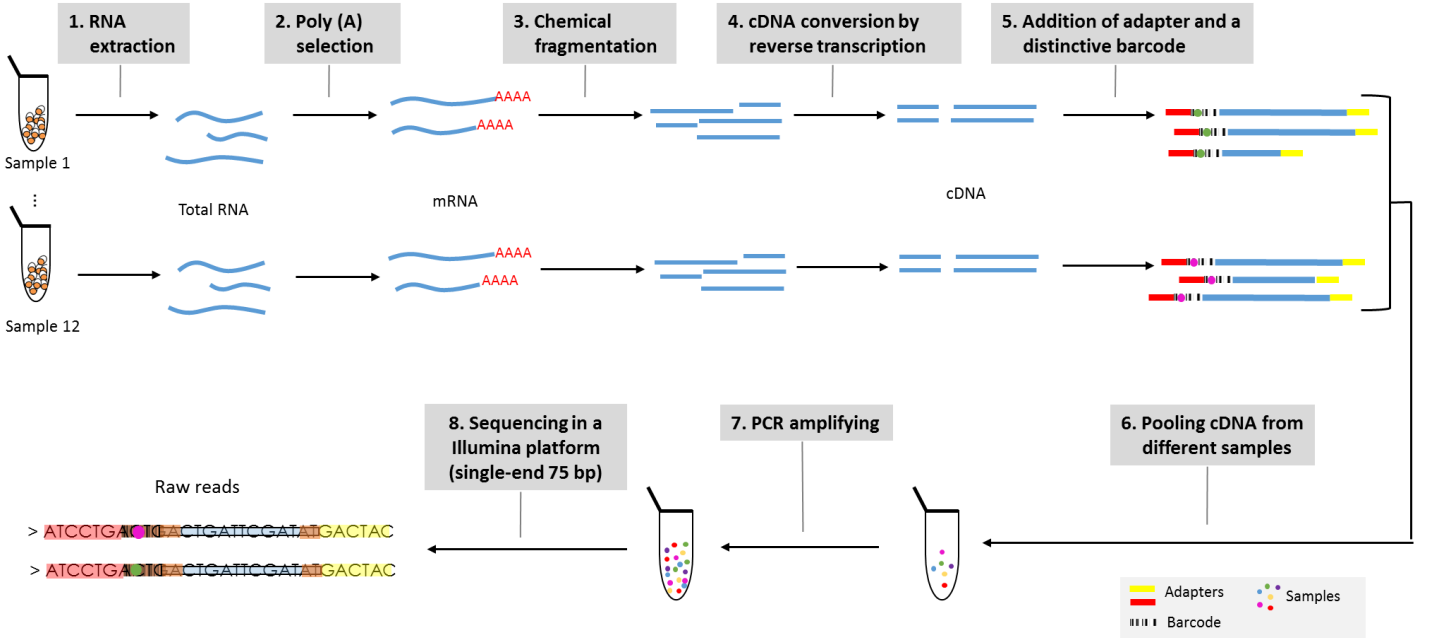


Figure 2.8. Scheme of the RNA-seq protocol followed for the 12 samples diapausing eggs produced by *Brachionus plicatilis* populations evolved under two regimes of environmental fluctuation (for details see the text in this chapter and Chapter 5).

Box 2.1. Glossary (modified from Franch-Gras 2017)

Adapter: specific oligonucleotides added to the DNA/cDNA fragments that match other oligonucleotides and are necessary for sequencing in most sequencing platforms.

Barcode: sequence that enables to identify the original sample from which the DNA information is obtained. This barcode is essential for samples to be distinguished and sorted during data analysis, since the DNA/cDNA from different samples is pooled prior to be amplified and sequenced together.

Complementary DNA (cDNA): the synthesis of DNA from a RNA template via reverse transcription produces cDNA, which could be used as a standard DNA.

DNA fragment: Fragment of DNA that is sequenced in which the order and identity of its bases is unknown.

DNA sequencing: process that allows determining the order and identity of the bases in a DNA fragment.

Functional annotation: process to associate biological information to the genomic elements structurally annotated (e.g., their biochemical and biological functions, etc.).

Gene ontology terms: descriptions of the known features of a gene according to its expression product classified in three domains of terms: (1) cellular component; (2) associated molecular function; and (3) associated biological process.

Multiplex sequencing: to process multiple DNA fragments at the same time in order to obtain their sequences.

Next-generation sequencing (NGS): type of DNA sequencing based on modern sequencing techniques (i.e., non-Sanger) yielding millions or billions of small reads from DNA fragments.

Paired-end sequencing (PE): a type of DNA sequencing which starts in both ends of the DNA.

Box 2.1. Glossary (continued).

Messenger RNA (mRNA): Is a type of RNA that serves as template for translation into proteins.

Read: each of the DNA sequences obtained from a DNA fragment.

Single-end sequencing (SE): a type of DNA sequencing which starts from only one end (i.e., extremity) of the DNA fragments.

Sequencing platform: Instrument that allows sequencing DNA.

Single Nucleotide polymorphism (SNP): variation in a single nucleotide locus between members of a species or paired chromosomes in an individual.

Structural annotation: physical regions of a genome containing a genomic element of interest such as genes, mRNA, repeated sequences, etc.

Transcriptomics: the study of all transcripts that are present at any time in the cell.

3

Evolution of life-history traits as a response to environmental unpredictability in experimental rotifer populations

Summary

The adaptive response of organisms to unpredictable environments is increasingly recognized as a central topic in fundamental and applied evolutionary ecology. Selection due to environmental unpredictability can act on multiple traits of an organism's life cycle to reduce the impact of high environmental variance. The aim of this chapter was to study how unpredictability selects for diapause traits: (1) the timing of sex (a proxy of the timing of diapausing egg production), and (2) the diapausing egg hatching fraction (inversely related to diapause duration). An experimental evolution approach with the facultative sexual rotifer *Brachionus plicatilis* was used. Laboratory populations experiencing two contrasting selective regimes (predictable versus unpredictable) evolved divergently over a short time span (< 77 days). The populations under the

unpredictable regime showed an earlier initiation of sexual reproduction and a lower hatching fraction of diapausing eggs than populations under the predictable regime. These findings empirically demonstrate the existence of bet-hedging adaptive responses in *B. plicatilis* regarding both traits, consistent with theoretical predictions of bet-hedging evolution under conditions of unpredictable environmental variance. Given that scenarios of increased environmental variability that are expected to occur in the near future, a comprehensive understanding of the role of bet hedging is necessary for predicting population responses to environmental change.

Introduction

Temporally varying environments are often characterized by an alternation between favorable and adverse periods (Chapter 1). However, such alternation is not always predictable. Theory predicts that organisms living in unpredictable environments might evolve bet-hedging responses to cope with environmental uncertainty (Cohen 1966; Seger and Brockmann 1987; Philippi and Seger 1989; Simons 2011).

To gain empirical evidence on bet hedging, both the use of isogenic lines and the performance of laboratory-controlled experiments have been considered as necessary (Simons 2011; Graham et al. 2014; Martínez-Ruiz and García-Roger 2015). As introduced in Chapter 2, experimental evolution has been recognized as a powerful tool in ecology and evolution for detecting short-term evolutionary responses under controlled conditions (Kassen 2002; Garland and Rose 2009). Moreover, this approximation has been successfully applied for the study of bet hedging in various organisms, such as microbes (Beaumont et al. 2009) or fungi (Graham et al. 2014), and seems promising in the case of small metazoans (e.g., nematodes; Dey et al. 2016).

In temperate regions, rotifer populations are temporary, even those inhabiting permanent ponds (García-Roger et al. 2006). They annually colonize the water column during the so-called planktonic

growing season, which starts with the hatching of diapausing eggs from sediment banks (Kotani et al. 2001; García-Roger et al. 2006; Chapter 2).

In unpredictable habitats, the survival of rotifer populations depends on the adjustments of their life history traits to the season-to-season variation in the length of the growing season (García-Roger et al. 2014). However, the association between life-history traits in rotifer populations and the degree of habitat unpredictability has not been tested yet. Given that diapause is the only way to survive unsuitable conditions between growing seasons, there are at least two key life-history traits in the rotifer life cycle in which bet hedging could have evolved: (1) the timing of sex (a proxy of the timing of diapausing egg production) and (2) the diapausing egg hatching fraction in a growing season (inversely related to the duration of diapause) (García-Roger et al. 2017). Further, these two traits might interact to reduce the risks associated with environmental unpredictability (Spencer et al. 2001).

Theoretical studies have proposed the timing of sex as an instance of conservative bet hedging in rotifer populations inhabiting unpredictable environments (reviewed in García-Roger et al. 2017). If the end of the growing season cannot be predicted by the rotifers, then shifting to sexual reproduction as soon as possible can avoid

the risk of unexpectedly short growing seasons (i.e., leading to a complete reproductive failure of a rotifer genotype). However, this strategy would be sub-optimal because a rotifer genotype producing diapausing eggs too early, while favorable conditions still prevail, would incur a cost (i.e., the investment in diapause results in a direct reduction of the current population growth rate; Serra and King 1999; Carmona et al. 2009; Montero-Pau et al. 2014).

Not all diapausing eggs hatch when favorable conditions occur (Chapters 1 and 2); instead, some of them remain viable in the sediments for longer periods (i.e., decades or even centuries; Kotani et al. 2001; García-Roger et al. 2006) forming egg banks (De Stasio 1989; Evans and Dennehy 2005). This response might be a case of diversified bet hedging if diapausing eggs showing different diapause durations are derived from the same genotype (Schröder 2005; Martínez-Ruiz and García-Roger 2015; Chapter 1). The optimum timing for hatching of diapausing eggs has been well studied from a theoretical perspective (Spencer et al. 2001; García-Roger et al. 2014), motivated by previous work on seed germination (Cohen 1966). According to theory, in predictable habitats, a large fraction of diapausing eggs is expected to hatch in the season following their production. Thus, hatchlings would be able to produce a new cohort of diapausing eggs during the growing season, and egg banks would be continuously renewed year after year. Instead, in unpredictable habitats, low –or even zero–

recruitment to the egg bank at the end of the growing season would occur if all diapausing eggs derived from a genotype hatched from the egg bank simultaneously in an unexpectedly short growing season. Therefore, if only a fraction of the diapausing eggs hatches, a rotifer genotype would ensure its presence in subsequent favorable periods and thereby avoid extinction (Brendonck and De Meester 2003; García-Roger et al. 2014; 2017).

The aim of this chapter is to test how unpredictable environments may select for traits related to diapause in populations of the rotifer *Brachionus plicatilis* using an experimental evolution approach. The trajectories of both the timing of diapausing egg production and the duration of diapause were monitored in laboratory populations subjected to two different environmental regimes (predictable vs unpredictable) through several growing cycles (equivalent to growing seasons) interrupted by periods of habitat unsuitability (see Chapter 2). In this chapter the prediction from bet-hedging theory that environmental unpredictability selects for an early timing of sex (i.e., early production of diapausing eggs) and intermediate hatching fractions in this rotifer species are tested.

Material and methods

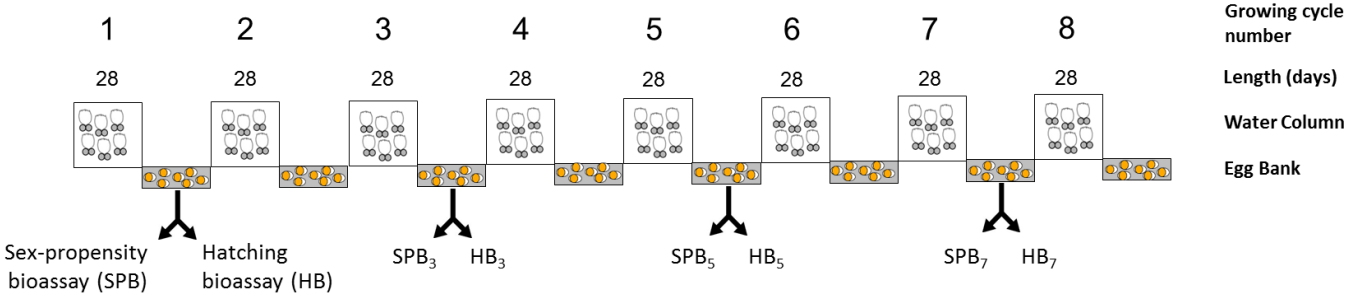
Laboratory populations, experimental evolution design and procedures

The establishment of the laboratory populations and experimental evolution design has been described in Chapter 2. Briefly, six laboratory populations having the same genetically diverse composition were randomly assigned to each of two experimental selective regimes under which they independently evolved through a series of eight growing cycles (see Figure 2.2). Three of them (working as replicates) were assigned to the predictable regime and the other three were assigned to the unpredictable one. Both experimental regimes followed a fluctuating pattern, but in one of them, the length of the growing cycles was kept constant (predictable), whereas it varied in the other (unpredictable). The end of each growing season was simulated experimentally by filtering the cultures –mimicking pond desiccation– and keeping the produced diapausing eggs dehydrated in the dark at 4 °C –allowing them to rest– during these simulated periods of habitat unsuitability (for details see Chapter 2). Like in the wild, the successive growing cycles were initiated with the hatchlings of diapausing eggs harvested from the previous growing cycle. In the predictable regime, the length of each growing cycle was always 28 days, whereas it varied among cycles in the unpredictable regime,

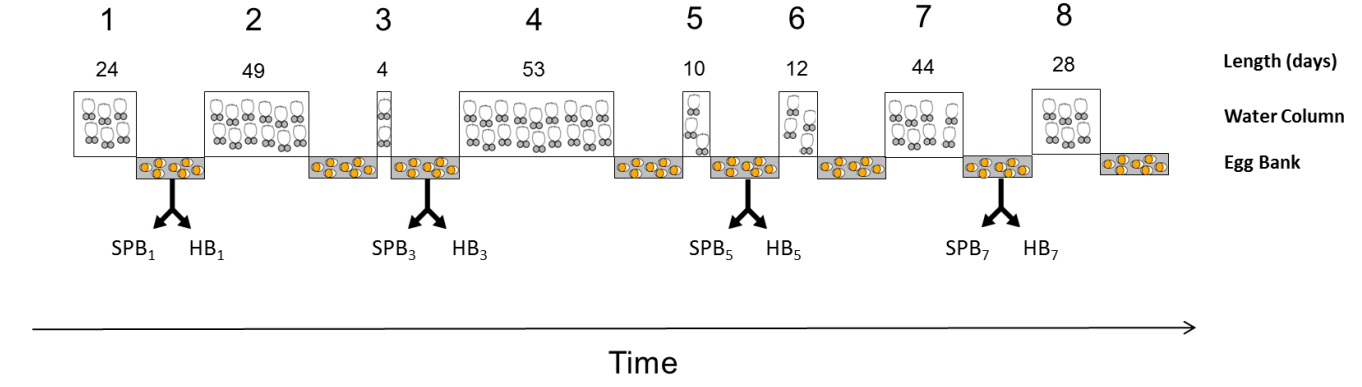
with the only constriction of averaging 28 days for the eight growing cycles. The duration of the unsuitable period (diapause) was 28 days in all cases. The two experimental regimes were carried out synchronously, involving a total of 448 days (growing cycles plus diapause periods; Figure 3.1).

Figure 3.1. Schematic diagram of the experimental evolution design. Growing cycles of the active population in the "water column" and diapause in the "egg bank" are displayed. The duration of the unsuitable period was 28 days in all cases. Arrows represent bioassays for estimation of the life history traits.

Predictable regime



Unpredictable regime



Life-history trait estimation

The life-history traits under study were estimated after cycles 1, 3, 5 and 7 (Figure 3.1). For each of the six populations, 70-200 diapausing eggs harvested from the corresponding growing cycle were individually placed in 96-multiwell dishes (Nunc™), induced to hatch (12 g L^{-1} of salinity, 25 °C and constant light), and monitored for six days. Once hatched, newborn females were transferred to Petri dishes with 40 mL of culture medium (250,000 cells mL^{-1} of *T. suecica* at 12 g L^{-1}) and allowed to proliferate asexually at 20 °C in darkness. From these clonal lines, ten were selected at random –as bioassay clones – and kept under the same conditions until the start of bioassays for life-history trait estimation. Two life-history traits were estimated in these clones: (1) the timing of sex –a measure inversely related to the density threshold for sex initiation (Carmona et al. 2009)–, and (2) the diapausing egg hatching fraction.

Timing of sex

Timing of sex was estimated in the bioassay clones. In total, 720 bioassays (= 6 populations x 10 clones x 3 replicates x 4 cycles) were performed to calculate the density threshold for sex initiation. The procedure followed Carmona et al. (2009) with minor modifications. Before each bioassay, rotifers were raised in pre-experimental cultures under standard conditions at low population

density (0.02-0.075 females mL^{-1}) and medium renewal for three generations. The purpose was to control for maternal effects (Stelzer and Snell 2006) and prevent sexual reproduction from already being induced at the onset of the bioassays. For each clone, three independent pre-experimental cultures were initiated by transferring single ovigerous females –each female typically carrying two eggs– to Petri dishes with 40 mL of fresh culture medium. After 24 hours, only one newborn female (F1) was kept in each plate, allowing her to asexually reproduce for the next 48 hours. Then, one of the newborn females (F2) was transferred into a new Petri dish with fresh culture medium. After 48 hours, each bioassay started by transferring a single newborn female (F3) to 15 mL of fresh culture medium (*T. suecica*, 500,000 cells mL^{-1}) and allowing the culture to proliferate. Cultures were checked every 12 hours until the first male was observed –sex initiation– (Aparici et al. 2001). Then, the culture was fixed with Lugol (4%, final concentration), and females were counted to estimate density. A lower population density threshold for sex initiation is indicative of an earlier timing of sex.

Diapausing egg hatching fraction

Hatching fraction was estimated using diapausing eggs produced by the same bioassay clones as for the previous life-history trait. To test for differences in the hatching fraction, it was essential to produce high numbers of diapausing eggs of equal quality and age.

For this purpose, the ten bioassay clones selected from each population and cycle (see above) were allowed to grow individually in Petri dishes containing 40 mL of culture medium. During the entire production period of diapausing eggs, food was supplied in excess (over 250,000 *T. suecica* cells mL^{-1}) by adding a suspension of concentrated microalgae (i.e., centrifuged at 1,500 rcf). Rotifer cultures were monitored for diapausing egg production daily and, once the first diapausing egg was observed in any clone, a period of four days was established for the production and maturation of diapausing eggs. On the fourth day, well-formed diapausing eggs (type I and II; García-Roger et al. 2005) were picked up and isolated individually in 96-multiwell plates (Nunc™) to be directly subjected to standard hatching conditions (6 g L^{-1} salinity, 25 °C and constant light; García-Roger et al. 2005; 2006). Diapausing eggs were incubated immediately after production to accurately record the timing of egg hatching. As it has been recently reported, the hatching of diapausing eggs can occur within a few days after production (Becks and Agrawal 2012; Martínez-Ruiz and García-Roger 2015). If fewer than 96 diapausing eggs were yielded by a bioassay clone, the cultures were maintained for as many rounds of four days as needed to achieve 96 eggs. The corresponding delay in production time was recorded. However, most of the clones (95.7%) produced the required 96 eggs after the first or second round.

Diapausing eggs were checked for hatching and deterioration at 24-hour intervals during 28 days. From these observations, the mean hatching fraction for each experimental regime at each cycle was estimated by averaging the hatching fractions of the clones from the three replicate populations, excluding deteriorated eggs (as in García-Roger et al. 2005). The viability of the unhatched fraction of diapausing eggs that remained healthy (García-Roger et al. 2005) was tested in a second round of incubation with similar conditions as above, after 28 days of additional dormancy.

Synchrony of diapausing egg hatching was estimated for each bioassay clone in the seventh growing cycle by calculating the IQR/H_{50} ratio as a measure of dispersion, where IQR is the interquartile range, and H_{50} is the median hatching time (i.e., time in days needed for 50% of eggs to hatch).

Data analyses

Generalized linear mixed-effect models (GLMMs) were used in order to test for an effect of experimental environment on the two life-history traits studied, and to compare between regimes across time. The assumed error distribution and link function depended on the life-history trait. The timing of sex (measured as the density for sex initiation) was analyzed using a Poisson distribution of errors and log for the link function. For the diapausing egg hatching fraction, a binomial distribution of errors and logit for the link

function were used. The structure of random and fixed effects was the same for both life-history traits. To explore the difference in effect between environmental regimes (predictable vs unpredictable) across cycles (1, 3, 5, and 7), a combined variable "Regime x Cycle" was used. This variable was treated as a factor of fixed effects with eight (2 regimes x 4 cycles) levels. In contrast, "Population" and "Clone" were random-effect factors. A nested design, with "Population" nested within "Regime x Cycle" and "Clone" nested within "Population" was used. Although the same populations were explored through different growing cycles, the inspection of the residuals did not show evidence of dependence. The significance of random effects was derived from likelihood ratio tests (LRTs) among nested models using Restricted Maximum Likelihood (REML; Bolker et al. 2009). Briefly, LRTs were used to compare the likelihood of models in which the random effect of interest (e.g., "Population" or "Clone") is included against reduced models without the effect in question. In each case, the conclusion was that the random effect was significant if the difference between the likelihood of the two models was significant as assessed by a χ^2 statistic (Pinheiro and Bates 2000).

Based on GLMMs, multiple planned comparisons to test for differences due to the experimental environment at each of the cycles of experimental evolution were performed. The alternative hypotheses were unidirectional (i.e., earlier timing of sex and lower

hatching fractions in the unpredictable regime than in the predictable one), and the *P*-values were adjusted accordingly. All analyses were carried out using R v. 3.2.2 (R Development Core Team 2015). The *glmer* and *anova* functions from the "lme4" package (Bates et al. 2015) for GLMMs and LRTs, respectively, were used. The function *glht* from the "multcomp" package (Hothorn et al. 2014) was used for the multiple planned comparisons.

Results

Diapausing egg bank dynamics

The net numbers of diapausing eggs produced at the end of each growing cycle (i.e., harvested eggs minus eggs used to re-found the populations) during the selection experiment are shown in Figure 3.2. An increase in net diapausing egg production across the cycles was observed under the predictable regime. In contrast, under the unpredictable regime, net diapausing egg production did not follow any particular trend, but fluctuated across growing cycles. In fact, the length of the growing cycles had a marked effect on diapausing egg production. A significant positive correlation was observed between production of diapausing eggs and length of growing cycle (R^2= 0.741, *P*-value= 0.012). As expected, in cycle 3 (lasting 4 days), there was no net diapausing egg production in any of the populations subjected to the unpredictable regime. In this cycle, no

diapausing egg-producing females were observed (data not shown) and only 80-150 diapausing eggs were harvested from the laboratory populations. These eggs most likely correspond to unhatched eggs remaining from those used to initiate the cycle.

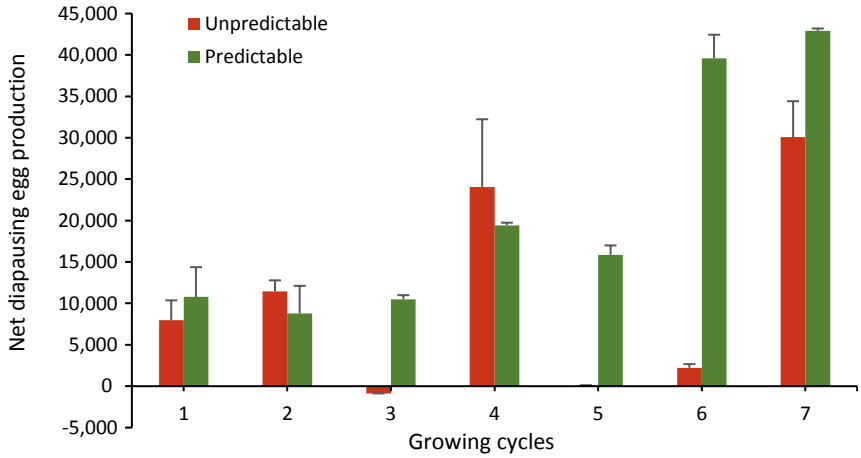


Figure 3.2. Net diapausing egg production at the end of each growing cycle under the selective regimes (predictable vs unpredictable). Bars are averages for populations within each regime (± 1 SE).

Evolution of the timing of sex

The timing of sex differed among the levels of the "Regime x Cycle" variable (*P*-value< 0.001; Table 3.1). The results from REML ratio tests for the significance of random effects in GLMM analysis did not reveal significant differences in the timing of sex among rotifer

populations within each combination of environmental regime and cycle (*P*-value= 0.554; Table 3.1), which means that the three replicated populations followed similar evolutionary dynamics within each regime. On the other hand, clones varied significantly within populations for this trait (*P*-value< 0.001; Table 3.1).

Table 3.1. Summary of the generalized linear mixed effects model (GLMM) on the timing of sex across the growing cycles analyzed. Fixed effects were tested using *F* tests. Random effects were tested through REML ratio tests by using a χ^2 statistic.

Effect	***d.f.***	***F*** [(a)]**/** χ^2 [(b)]	***P*-value**
Regime x Cycle	7, 35	12.92 [(a)]	< 0.001
Population (Regime x Cycle)	1*	0.35 [(b)]	0.554
Clone (Population (Regime x Cycle))	1*	22,134 [(b)]	< 0.001

*Degrees of freedom for REML ratio tests comparing nested models with and without the factor under study.

When comparing regimes at each individual growing cycle for the timing of sex, significant differences in the seventh cycle were detected (*P*-values in Figure 3.3). In this last cycle, the average densities for sex initiation were 8.1 and 13 females mL^{-1}, respectively, for the unpredictable and predictable regimes.

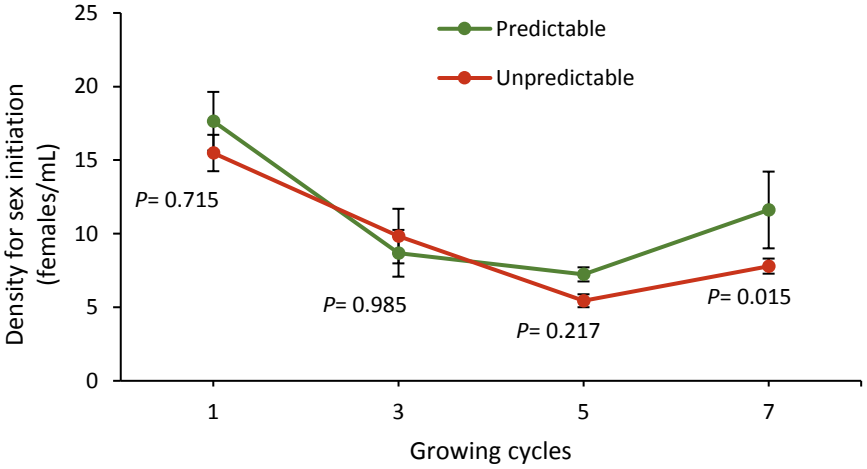


Figure 3.3. Evolutionary progression of the density for sex initiation (i.e., a proxy for the timing of sex) in two different experimental regimes. Mean (± 1 SE) population densities for sex initiation after the growing cycles 1, 3, 5 and 7 are shown. *P*-values for planned comparisons between selective regimes are shown.

Evolution of the diapausing egg hatching fraction

Rotifers evolved different diapausing egg hatching fractions depending on the selective environment, unpredictable vs predictable (Figure 3.4). The GLMM analysis (Table 3.2) revealed a significant effect of the "Regime x Cycle" factor (*P*-value< 0.001). Cycle-by-cycle comparisons between regimes demonstrated significant differences in the hatching fraction of diapausing eggs, except in cycle 1 (*P*-values; Figure 3.4). Populations under the unpredictable regime showed lower hatching fractions (being

58.2%, 50.8% and 64.7% for cycles 3, 5 and 7, respectively) compared to populations under the predictable regime (hatching fractions being 87.6%, 90.1% and 92.7%, respectively, for the same cycles).

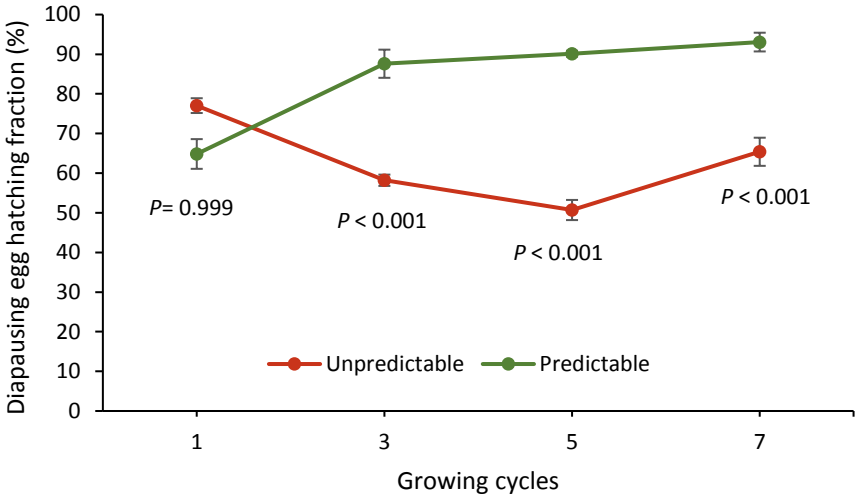


Figure 3.4. Evolutionary progression of the hatching fraction in two different experimental regimes. Mean (± 1 SE) hatching fractions of diapausing eggs after the growing cycles 1, 3, 5 and 7 are shown. *P*-values for planned comparisons between selective regimes are shown.

Results from REML ratio tests for random effects did not reveal significant differences in the hatching fraction of diapausing eggs among rotifer populations within each combination of selective regime and cycle (*P*-value> 0.999; Table 3.2), which again indicates that populations evolved in the same way within each regime. On the other hand, statistically significant differences in the hatching

fraction of diapausing eggs produced by different clones within populations were found (*P*-value< 0.001; Table 3.2), which shows the continued presence of genetic variation within the populations under selection after seven cycles of growth.

Table 3.2. Summary of the generalized linear mixed effects model (GLMM) on diapausing egg hatching fraction across the growing cycles analyzed. Fixed effects were tested using *F* tests, whereas random effects were tested through REML ratio tests by using a χ^2 statistic.

Effect	***d.f.***	***F*** [(a)] **/** χ^2 [(b)]	***P*-value**
Regime x Cycle	7, 35	21.337 [(a)]	< 0.001
Population (Regime x Cycle)	1*	0 [(b)]	> 0.999
Clone (Population (Regime x Cycle))	1*	2325.4 [(b)]	< 0.001

*Degrees of freedom for REML ratio tests comparing nested models with and without the factor under study.

The relationship between diapausing egg hatching fraction and H_{50} showed a pattern of delayed hatching in the populations evolved in the unpredictable regime (Figure 3.5). The IQR/H_{50} ratio averaged 0.87 ± 0.08 and 0.56 ± 0.08, respectively, in the predictable and unpredictable regimes.

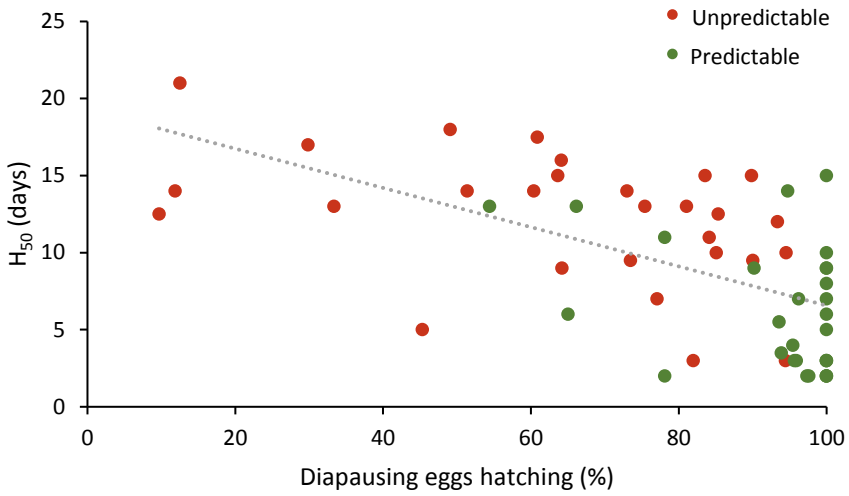


Figure 3.5. Relationship between hatching fraction of diapausing eggs and H_{50}. Dots represent rotifer clones (n= 56) from cycle 7. Dotted line represents least-square regression (R^2= 0.3649; P-value< 0.05).

Discussion

Theory predicts that the evolution of bet hedging underlies adaptive responses to environments under conditions of unpredictable environmental variance (Cohen 1966; Seger and Brockmann 1987; Philippi and Seger 1989; Simons 2011). In the comparative approach carried out in this chapter for the assessment of bet hedging in diapause-related traits, it was found that –in a manner consistent with theoretical expectations– in the populations under the unpredictable regime, there was an earlier initiation of sexual reproduction and a lower hatching fraction of

diapausing eggs than in the populations under the predictable regime.

This chapter shows that rotifer populations rapidly responded to a particular unpredictable fluctuation pattern under experimental evolution. This strongly supports that rotifers can rapidly adapt to unpredictable environments. Diapause-related traits evolved divergently in the laboratory populations under the two contrasting selective regimes over a short time period. Such a rapid evolution was remarkable for the hatching fraction, a trait that showed significant divergence after three growing cycles (i.e., 77 days for the unpredictable regime and 84 days for the predictable one). Asexual and sexual generations in the cultures were not tracked, but their numbers can be roughly estimated from previous observations (see Material and methods). Hence, ca. 17-18 asexual and six sexual generations may have occurred when statistically significant divergence was detected for the first time between selective regimes for the hatching fraction (cycle 3). In the case of the timing of sex, significant differences between both regimes were detected in the last growing cycle assayed (i.e., 196 days). This accounts for ca. 44 asexual and 18 sexual generations in the unpredictable regime and ca. 42 asexual and 14 sexual generations in the predictable one. These results are consistent with previous studies on micro-evolutionary adaptation using rotifers as model organisms, which have demonstrated evolutionary change in very

short periods (< 200 days), providing solid evidence of their high ability for rapid adaptation (reviewed in Declerck and Papakostas 2017; Walczyńska et al. 2017).

The experimental evolution protocol used in this thesis mimicked selection imposed by habitat unpredictability in natural rotifer populations. Water bodies of temperate latitudes are rotifer habitats exposed to environmental variability, often characterized by season-to-season fluctuations in the length of the growing season (García-Roger et al. 2014) and becoming unsuitable for periods of varying predictability (Ortells et al. 2000). In these habitats, rotifer populations can be conceived as a collection of clones (i.e., asexual descendants of a single genotype) that reproduce sexually to produce diapausing eggs (Gómez and Carvalho 2000), some of them hatching in the next growing season. Since survival of a given genotype to the next growing season is only possible through diapausing eggs, the total number of diapausing eggs produced is considered a between-season fitness measure (Serra and King 1999; Campillo et al. 2011). Genotypes failing to reproduce sexually do not contribute diapausing eggs to the bank and consequently have zero fitness. In the experiments performed in this thesis, regular-term growing cycles (predictable regime) allowed a steadily increasing production of diapausing eggs across growing cycles. Conversely, diapausing egg production in the unpredictable regime varied dramatically depending on the length

of the cycle, and most likely no eggs were produced in the shortest cycle (4 days in cycle 3). The recovery of the populations under the unpredictable regime after this cycle highlights the importance of the egg bank to ensure the persistence of populations (Brendonck and De Meester 2003). Fluctuations in the length of the growing cycle under the unpredictable regime provided strong selective pressure driving the rapid divergent evolution observed in the rotifer populations despite bottlenecks occurring after shorter cycles. The assayed sequence of cycle lengths in the unpredictable regime would have helped this rapid divergence, as the resultant fluctuation was already high in the first three cycles (24, 49 and 4 days in length, respectively). Even so, the cycle lengths in this sequence may not be equally effective in selecting for both traits. For instance, during cycle 3, a growing period as short as 4 days – likely not allowing any genotype to complete sexual reproduction– might have had no effect on the selection for early sex.

A caveat to this outcome of rapid divergence, however, is that the rate of evolution may have been enhanced in the evolution experiments compared to nature. Although genetic variation for ecologically relevant traits is ubiquitous in natural populations (Aparici et al. 2001; Carmona et al. 2009; Campillo et al. 2011; Gabaldón and Carmona 2015), the genetic diversity of the starting laboratory populations was experimentally increased by mixing clones founded with field-collected sexual eggs from a set of natural

populations, so the genetic variation from which to select during the experiment was high (Chapter 2). Moreover, these clones likely have distinct ecological features because the source populations covered a wide range of habitat predictability (Franch-Gras et al. 2017a; 2017b); consequently, some of the genotypes may have been pre-adapted to either of the two selective regimes assayed, fueling rapid adaptation.

Early sex initiation has been shown to evolve rapidly in previous experimental evolution studies with rotifers under a variety of selective factors (e.g., homogenous vs heterogeneous food environments, Becks and Agrawal 2010; maintained vs novel environments, Becks and Agrawal 2012; and permanent vs seasonal growing seasons, Smith and Snell 2012). However, the relationship between the timing of sex and environmental unpredictability has not been assessed until now.

After seven cycles of divergent evolution, it was observed that rotifers initiated sexual reproduction earlier than expected –if compared to the average cycle length– under the unpredictable regime, which can be interpreted as an instance of conservative bet hedging. As stated in Chapter 1, temporal environmental variation might also result in other responses such as phenotypic plasticity, or adaptive tracking (Bell and Collins 2008; Simons 2011). Phenotypic plasticity is expected to occur when environmental cues

are good predictors of future conditions (Furness et al. 2015), which is not the case in this experiment. Notably, the fixation of the long-term optimal strategy could be partially slowed down during the clonal growing phases by an adaptive tracking process (Bell and Collins 2008), as each growing cycle could act as a selective regime for earlier or delayed sex (Carmona et al. 2009). Moreover, sexual reproduction –by producing maladapted genotypes– may result in a genetic slippage effect in the evolution of the trait (i.e., a change in the mean genotypic value contrary to that promoted by selection; Lynch and Deng 1994), thus requiring more growing cycles to reach the optimal value.

The unpredictable regime drove divergent evolution of bet hedging in diapause duration, as indicated by the significant differences observed in the hatching fraction between the two regimes across the cycles of experimental evolution (ca. 30% difference in hatching fraction between regimes at the end of the experiment). The populations subjected to the unpredictable regime evolved longer diapauses (i.e., lower hatching fractions) than the populations subjected to the predictable regime. This finding is interpreted as an instance of diversified bet hedging (i.e., a single genotype produces different phenotypes in its offspring in advance of future unpredictable conditions) because not all diapausing eggs produced by a clone hatched at the same time (Schröder 2005; Jong et al. 2011). In the predictable regime, the hatching fraction

progressively increased from 65% in cycle 1 to ca. 93% in cycle 7. However, a decrease in the hatching fraction was observed in the unpredictable regime, from 77% in cycle 1 to ca. 65% in cycle 7. These results are qualitatively consistent with the predictions from Cohen's (1966) classic "good vs bad season" bet-hedging model and derivatives (García-Roger et al. 2014) because evolved hatching fractions were proportional to the frequency of "good" (i.e., net production of diapausing eggs > 0) growing seasons.

A striking result from this study is that clones from populations evolved in an unpredictable regime exhibited a more synchronous pattern in the hatching of "early" hatchers than the clones from the predictable regime. This means that once a new growing season has started, hatchlings in the predictable regime are spread throughout the season. Such asynchrony has been described elsewhere as a kind of within-season bet hedging (Vanoverbeke and De Meester 2009). This strategy may allow risk-aversion if the occurrence of successful growing seasons is predictable but there is some uncertainty with respect to their start, as may occur in the source natural populations of the genotypes used in this experiment. Because this condition was not tested here, it was hypothesized that this phenomenon occurred to either a pleiotropic effect or linkage between genes associated with the life-history traits under study.

A thorough search of the relevant literature has yielded only three studies on experimental evolution that addressing selection of bet-hedging strategies in temporally varying environments using predictable and unpredictable regimes (Beaumont et al. 2009; Graham et al. 2014; Dey et al. 2016). Graham et al. (2014) observed the evolution of intermediate germination fractions under successive cycles of unpredictable selection in the fungus *Neurospora crassa*. Moreover, parental effects have been highlighted in the nematode, *Caenorhabditis elegans,* as a cause of adaptation to fluctuating environments (Dey et al. 2016). Interestingly, diversified bet hedging in the hatching fraction might be caused by randomizing factors related to the physiological age of the mother (Marshall and Uller 2007; Martínez-Ruiz and García-Roger 2015), or other parental effects affecting the amount of reserves allocated to diapausing eggs (e.g., mother size), or their sensitivity to hatching cues (Pinceel et al. 2013). Alternatively, variable diapause duration at the within-clutch level could result from an adaptive "coin-flipping" mechanism (Childs et al. 2010), as for instance due to random microenvironmental conditions during embryo development (Simons and Johnston 2003).

The achieved level of empirical evidence in favor of bet hedging in the present study was high, since level V of Simon's six assessment criteria (2011) was fulfilled in both life-history traits assayed (Table 3.3). This level was achieved because the traits under fluctuating

selection evolved in the direction predicted by well-established theoretical models (Serra and King 1999; Spencer et al. 2001; García-Roger et al. 2014). No empirical test was performed for IV, which was assumed given that levels of evidence are inclusive (Simons 2011). To the date, there are no empirical data directly supporting this level, but fitness consequences of the traits can be inferred from the following verbal argumentation based on the predictions raised by the above mentioned models. One can simply plead for an educated guess on the fitness outcome (i.e., diapausing egg production) of the evolved values of the traits when subject to each other regime. Hence, a rotifer clone selected under the predictable regime, with late production of diapausing eggs and hatching fractions close to 100%, may result in zero fitness if subject to an unexpectedly short growing season as in the unpredictable regime. However, such a combination of trait values is easily recognized to perform better in the predictable regime than an early production of diapausing eggs and intermediate hatching fractions as evolved in rotifer clones from the unpredictable regime (see Figure 3.2). While most studies on bet hedging in diapause-related traits typically hold intermediate levels of evidence (i.e., level III; see García-Roger et al. 2017), the present study of the duration of diapause goes further and represents a strong line of evidence for diversified bet hedging in metazoans (Simons 2011).

Table 3.3. Levels of evidence of bet hedging in the timing of sex and diapausing egg hatching fraction in *Brachionus plicatilis*. Level description modified from Simons (2011). Plus (+) and minus (-) symbols indicate whether the criteria are fulfilled or not.

Level	Description	Timing of sex	Hatching fraction
I	A low-risk (for conservative bet hedging) or variable (for diversified bet hedging) trait was identified	+	+
II	An environmental factor leading to habitat unpredictability was tested	+	+
III	Variation in the trait differed among populations	+	+
IV	Fitness consequences of the trait assayed in ≥ 2 contrasting regimes[a]	+	+
V	Trait under fluctuating selection evolved in the predicted direction	+	+
VI	Trait evolved to the long-term optimal strategy according to the degree of unpredictability	-	-

[a] This level was fulfilled based on a verbal argumentation and theoretical models (see text).

In this Chapter, using an experimental evolution approach, it has been empirically demonstrated that *B. plicatilis* can evolve bet-hedging strategies in two diapause-related traits as a response to environmental unpredictability. Both, early production of diapausing eggs and a low hatching fraction serve the same objective of avoiding the risk of a complete reproductive failure in habitats with an unpredictable length of the growing season. However, because these two traits have the same role, it may be interesting to ascertain whether selection of both traits is possible over the long term or whether one should evolve instead of the other (Wilbur and Rudolf 2006). Recent theoretical work has suggested that organisms hedging their bets successfully with one strategy do not need to bet hedge as much with another (Spencer et al. 2001); thus, a trade-off between these two traits would be expected to evolve (Starrfelt and Kokko 2012a). Although it has been observed that both strategies can be selected simultaneously, further research implementing more growing cycles will be needed to provide a definitive response to this question.

Uncertainty is an inherent feature of natural environments, and this study demonstrates that organisms can develop fast evolutionary responses to address environmental unpredictability. Given that scenarios of increased environmental variability are expected to occur in the near future (IPCC 2013), the persistence of natural populations under these circumstances may depend on the

evolution of bet hedging in key life-history traits (Childs et al. 2010, Simons 2011; Gremer and Venable 2014). Therefore, a comprehensive understanding of the role of bet hedging is necessary for predicting population responses to environmental change (Lawson et al. 2015). This study provides an important first step in this direction, by demonstrating that the evolution of bet-hedging strategies can occur rapidly, at ecological timescales. However, understanding how these strategies interact and unraveling the molecular mechanisms underlying response variation will increase the knowledge of how organisms adapt to unpredictable environments.

4

Ecological genomics of adaptation to unpredictability in rotifer experimental populations

Summary

Elucidating the genetic basis of phenotypic variation in ecologically relevant traits in response to different environments is key to understand how populations evolve. Facultatively sexual rotifers can develop adaptive responses in their life history traits in response to temporally varying environments. In Chapter 3, laboratory populations of the rotifer *Brachionus plicatilis* showed rapid changes in life-history traits related to diapause in response to two selective regimes (predictable vs unpredictable). Here, the genomic basis of adaptation to environmental unpredictability of these populations is unravelled. Genome-wide polymorphisms in 169 clones from both selective regimes at the end of the seventh cycle of experimental evolution were identified and genotyped using genotyping by sequencing (GBS). In addition, published GBS

data from 270 field clones –those used to establish the laboratory populations– and a draft genome assembly were used. This large GBS dataset was exploited to identify outlier loci as candidates to be under selection in the predictable and unpredictable regime. Three candidate SNPs were identified to be under selection with respect to unpredictable environments and a strong signal of adaptation to laboratory conditions was observed. Additionally, a genotype-phenotype association approach revealed five SNPs associated with two life-history traits related to diapause that are key in the adaptation to unpredictable environments: timing of sex and diapausing egg hatching fraction (Chapter 3). This study provides key information for (1) elucidating the genomic basis for adaptation to unpredictable environments, and (2) laying the groundwork for future studies that will provide a better understanding of the genome and transcriptome of *B. plicatilis*.

Introduction

To unravel the genetic mechanisms underlying organism's phenotypic variation is essential to understand the adaptive responses to temporally varying environments (Storz et al. 2005; Stinchcombe and Hoekstra 2008). Anthropogenic effects and climate change have been identified as amplifiers of environmental unpredictability and the expectation is that these impacts will increase even more in the near future (IPCC 2013). Research is needed to know whether these adaptations will be enough to cope with the upcoming increase in environmental unpredictability. Consequently, (1) understanding organisms' evolutionary responses to unpredictability, and (2) unraveling the molecular mechanisms and the genomic basis underlying these adaptations are key issues in evolutionary and conservation biology.

As stated in Chapter 1, different adaptive responses (i.e., adaptive tracking, phenotypic plasticity and bet hedging) have been described in organisms that inhabit temporally varying environments (Philippi and Seger 1989; Reed et al. 2010; Simons 2011; Tufto 2015). Despite the relevance of the ubiquitous challenge posed by environmental fluctuation and the diversity of adaptive responses displayed by organisms, empirical studies elucidating the genomic basis of these responses are still scarce.

Experimental evolution has been considered a powerful tool to explore the genomic basis of adaptation (Barrett and Hoekstra 2011; Matos et al. 2015). In this type of experiments, generally populations derived from a single ancestral genotype –i.e., a genetically homogeneous population– are exposed to different selective regimes (Kawecki et al. 2012; Teotónio et al. 2017). However, other studies use a combination of several genotypes or populations –i.e., several ancestral genotypes or a polymorphic population– to establish the experimental populations. The evolutionary outcomes are expected to differ depending on whether the experimental populations are established from a single or from multiple genotypes. If the founding population is genetically identical, adaptation to selective regimes could happen through the accumulation of beneficial mutations. In contrast, if the founding population is polymorphic –and given the relative short-term scale of evolution experiments– selection is expected to act on the heritable standing variation (Barret and Schluter 2008; Hohenlohe et al. 2010; Schlotterer et al. 2015), which has been predicted to lead to rapid evolution in novel environments (Hermisson and Pennings, 2005).

However, the divergence between populations subjected to different selective regimes will be driven not only by selection, but also by random genetic drift, which could be important if selective regimes favor bottleneck events and reduce effective population

size (Heffernan and Wahl 2002; Li and Roossinck 2004). For this reason, population replicates evolving independently under the same selective regime are essential to test evolutionary hypotheses. A consistent response across experimental population replicates could allow differentiating between stochastic and deterministic events (Schlotterer et al. 2015; Long et al 2015; Jha et al. 2015), increasing the power to draw meaningful conclusions about the adaptation to the tested conditions.

The rotifer *Brachionus plicatilis* has been shown to be a good model organism to test evolutionary hypotheses using experimental evolution approaches (Chapter 2). As seen in Chapter 3, *B. plicatilis* laboratory populations showed rapid adaptation to unpredictable environments, displaying a divergent response in two life-history traits (i.e., the timing of sex and the diapausing egg hatching fraction). Populations subjected to the unpredictable selective regime showed both lower hatching fractions of diapausing eggs and earlier sex initiation, suggesting that bet hedging strategies underlie adaptation to environmental unpredictability in these organisms (see Chapter 3). In natural populations, Franch-Gras et al. (2017b) found signatures of local adaptation to the degree of environmental predictability of their habitats in both the timing of sex and the diapausing egg hatching fraction. Interestingly, high genetic variance related to these traits has been found in field populations of *B. plicatilis* (Campillo et al. 2009; Franch-Gras et al.

2017b). However, despite the progress identifying adaptive responses in rotifer life-history traits to environmental unpredictability (Chapter 3; Franch-Gras et al. 2017b), studies on the genetic basis underlying these responses are almost non-existent.

Identifying genetic signatures of selection at the genome level in wild and/or experimental populations is key to elucidate the genetic basis of ecological adaptation. Next-generation sequencing (NGS) techniques have made possible to study genetics of adaptation in non-model species (Van Straalen and Roelofs 20011; Stapley et al. 2010). One of such techniques, genotyping by sequencing (GBS; Elshire et al. 2011), relies on the use of restriction enzymes to reduce the genome complexity (Chapter 2). This technique allows to identify and genotype of thousands of single nucleotide polymorphisms (SNPs) in individuals from different populations (reviewed in Davey et al. 2011; Narum et al. 2013; Chapter 2). Moreover, the genome locations with signatures of selection can be detected (Stapley et al. 2010). There are several methods that aim to detect loci under selection. Among them are the F_{ST}-based methods that compare the allele frequencies among populations to identify those SNPs that have higher differentiation values than those expected under a neutral evolution model (Beaumont and Balding 2004, Foll and Gaggiotti 2008). Recently, other methods such as GWAS (genome-wide association studies)

have been developed to detect signatures of selection. These methods are based on the association of the phenotypic trait variations with their genotype at the individual level (Goodard and Hayes 2009).

In this chapter the genomic basis of adaptation to environmental unpredictability was studied in *B. plicatilis*. By using GBS, hundreds of markers were identified and genotyped in laboratory *B. plicatilis* populations that evolved in two contrasting selective regimes (predictable vs unpredictable; Chapters 2 and 3). Additionally, the newly available genome and the GBS data from nine *B. plicatilis* field populations (Franch-Gras et al. in review) –from which the initial laboratory populations were founded– were used in the analyses. This large GBS dataset (origin and experimental GBS data) was used to identify candidate loci for diversifying selection under these selective regimes.

Material and methods

Experimental laboratory populations and study clones

The set of clones analyzed was obtained at the end of the seventh cycle of the evolution experiment described in Chapter 2. Briefly, in that study six genetically identical laboratory populations of the rotifer *B. plicatilis* were founded from a pool of 270 clones (30

clonal lines from each of nine Spanish Mediterranean salt ponds and lakes; Franch-Gras et al. 2017b). These populations were subjected to two contrasting selective regimes (predictable vs unpredictable). Three of them were assigned to the predictable regime and the other three to the unpredictable one. By including several replicate experimental populations within each regime in the experimental evolution design, it was possible to evaluate their independent evolutionary trajectories.

In order to establish the rotifer clones to carry out DNA analyses (see below), 192 diapausing eggs per laboratory population were collected at the end of the seventh cycle of selection (see Figure 2.6), and after a forced period of dormancy (28 days), placed in 96-multiwell dishes (Nunc™) and induced to hatch under constant light, 12 g L^{-1} salinity and 25 °C (García-Roger et al. 2006). These eggs were monitored daily during 7 days and once hatched, newborn females were transferred to Petri dishes with 40 mL of f/2 saline water medium at 12 g L^{-1} (Guillard and Ryther 1962) containing 250,000 cells mL^{-1} of the microalgae *Tetraselmis suecica* as a standard culture medium. Females were allowed to proliferate asexually at 20 °C in darkness, thus establishing clonal lines. Overall, from each of the six laboratory populations, 30 randomly chosen clones were sampled (180 clones in total) and their DNA extracted.

To obtain enough biomass for DNA extractions, each individual rotifer clone was grown under standard culture medium (see above) in 1.5 L plastic bottles during 15 days, starting from a low-density stock culture. On day 15, when more than 15,000 individuals were reached in each bottle, cultures were filtered out through a 30-µm Nytal mesh sieve. The retained rotifers were released and kept on saline water 12 g L^{-1} for 24 hours in order to purge their digestive tract and minimize contamination with microalgae DNA. Thereafter, rotifers were retained in a 30-µm Nytal mesh sieve, which was frozen in liquid nitrogen. DNA extraction was performed on the frozen rotifer biomass using JETFLEX Genomic DNA purification kit (GENOMED, Löhne, Germany) following manufacturer's instructions. DNA quality was assessed on 1% agarose gel and the quantification of DNA was performed with a Qubit 2.0 fluorimeter (Life Technologies). A total of 169 clones were obtained with a good enough DNA quality and high enough concentration (> 20 ng/µl of DNA) to apply the genotyping-by-sequencing protocol.

Genotyping by sequencing (GBS)

GBS libraries were prepared and sequenced at the Institute for Genomic Diversity (IGD, Ithaca, NY, USA) following the protocol described by Elshire et al. (2011). In brief: (1) DNA samples were digested with the restriction enzyme *ApeKI* (GC[A-T]GC), (2) GBS

libraries were constructed with unique barcodes for each clone, and (3) after pooling of the samples, sequencing was carried out using an Illumina HiSeq 2000/2500 (100 bp, single-end) (see GBS protocol in Chapter 2).

SNP calling and filtering

Raw sequence data quality was analysed using FastQC version 0.11.5 (www.bioinformatics.babraham.ac.uk/projects/fastqc/). SNPs were called from the raw DNA sequences using the SNP calling v.2 pipeline as implemented in TASSEL 5 (Glaubitz et al. 2014). To apply this pipeline, a draft version of *B. plicatilis* genome (Franch-Gras et al. in review) was used. Additionally, the available GBS data (Franch-Gras et al. in review) from the 270 clones used as founders of the experimental laboratory populations (hereby origin population) were downloaded and employed for comparison. The default parameters from the TASSEL-GBS pipeline were used with some modifications: the minimum length of aligned base pair (-aLen 30) and the minimum locus coverage (-mnLCov 0.8).

The set of SNPs were filtered by quality using user developed scripts and VCFtools (Danecek et al. 2011). The following parameters were set: (1) a minimum coverage of six reads to call a genotype, (2) the presence of the SNP in at least 50% of individuals in each population, (3) minimum allele frequency (--maf) higher than 1%,

(4) only two alleles present (--min-alleles, --max-alleles), (5) average read depth < 150 (--max-meanDP), and (6) heterozygotes in each SNP < 60%. In general, these modifications were intended to follow a conservative approach in the detection of SNPs.

Data analysis

Genetic structure

To assess genome-wide genetic variation and differentiation, a principal component analysis (PCA) was performed using the *adegenet* package (Jombart 2008) of R v. 3.2.2 (R Development Core Team 2015). To evaluate between-population differentiation, the fixation index (F_{ST}) for each pairwise comparison was estimated. Moreover, expected heterozygosity (*He*), observed heterozygosity (*Ho*), and the inbreeding coefficient (F_{IS}) for the origin and the six experimental laboratory populations were estimated using the R package "hierfstat" (Goudet 2005). Hardy-Weinberg equilibrium (*HWE*) was tested using VCFtools for each population. File format conversions were performed using PGDSpider (Lischer and Excoffier 2012) and Plink v. 1.9 (Purcell et al. 2007).

Candidate SNPs under selection

Loci putatively under selection were identified by means of BayeScan version 2.1 (Foll and Gaggiotti 2008). This genome-scan method identifies markers that show divergence patterns between

groups that are stronger than would be expected under neutral genetic processes. Moreover, it estimates the posterior probability that a given SNP is affected by selection. This is a F_{ST}-based method that has been shown to be the most robust to differentiate confounding demographic processes (Pérez-Figueroa et al. 2010; De Villemereuil and Gaggiotti 2014) and to reduce false positives (Narum and Hess 2011). To do that comparison, the following groups were established: (1) origin population, (2) populations evolved under the predictable environmental fluctuation regime (hereafter predictable populations), and (3) populations evolved under the unpredictable environmental fluctuation regime (hereafter unpredictable populations). In order to assess the assignment of candidate SNPs to selective regimes (predictable vs unpredictable), as opposed to adaptation to general laboratory conditions during the experiment, a new BayeScan analysis using only two groups: (1) predictable populations and (2) unpredictable populations, was performed. So, each of the populations acting as replicates for each selective regime were assigned to the corresponding unpredictable or predictable group. Prior odds (PO) of 10 and false discovery rate (FDR) of 0.05 were used for identifying candidate SNPs.

Genome-wide association

Genotype-phenotype association analysis was performed for the two life-history traits –timing of sex and hatching fraction of

diapausing eggs– estimated for each clone in the previous chapter (Chapter 3). This analysis was performed in the clones in which both phenotypic and genotypic data were available (52 and 76 clones for hatching fraction and timing of sex, respectively). The SNPs data set was filtered for minimum allele frequency lower than 0.01. Two approximations to GWAS, Plink version 1.9 (Purcell et al. 2007) and GenABEL (Aulchenko et al. 2007) were used. In both of them a minimum allele frequency (maf) value of 0.01 was used and the *P*-values were adjusted for Bonferroni correction. Finally, linkage disequilibrium (LD) was assessed for those candidate SNPs associated with both phenotypic traits using Plink.

Identification of candidate genes under selection

Gene functions were retrieved from the current annotation of the *B. plicatilis* genome (Franch-Gras et al. in review). Candidate SNPs identified by BayeScan and those identified by GWAS were scanned in order to identify the putative genes associated using BEDtools (Quinlan and Hall 2010). Flanking regions of 0, 2.5 and 5 Kb, upstream and downstream, from the focus SNP were used. Additionally, a gene ontology (GO) enrichment analysis (see Box 2.1) was performed for those genes in which candidate SNPs under selection were found using Blast2GO (Conesa et al. 2005).

Results

GBS raw data, SNP calling and filtering

A total of 19 Gb of GBS raw data were obtained from 169 clones of the six laboratory populations. Out of the ca. 915 million raw reads from origin population plus the six laboratory populations, ca. 560 million reads were retained after quality filtering. After SNP calling and additional SNPs filtering, 6,107 SNPs were identified and genotyped. These filtered SNPs were used in downstream analyses (Table 4.1).

Table 4.1. Summary of SNP calling and filtering from the origin population plus the six laboratory populations. Numbers in brackets refer to filtering criteria in VCFtools (see Methods).

Raw reads	915,507,331
Quality-filter reads	506,614,399
SNP calling	
1-TASSEL pipeline	17,042
2-SNP filtering (1) and (2)	10,986
3-SNP filtering (3), (4) and (5)	6,424
4-SNP filtering (6)	6,107

Genetic structure

The principal component analysis (PCA) showed that clones from the origin population (Figure 4.1) –belonging to nine field rotifer populations– were widespread in the ordination space. The six laboratory populations overlapped in the center of the PCA ordination space (Figure 4.1), with a mean F_{ST} of 0.015 between selective regimes. Populations subjected to the predictable regime showed lower differentiation patterns (Figure 4.1 and Figure A.1) with their F_{ST} ranging from 0.016 to 0.025. Populations under the unpredictable regime were more dispersed from one another (Figure 4.1 and Figure A.1), with F_{ST} ranging from 0.046 to 0.078. Pairwise F_{ST} values between the origin and the six laboratory populations are shown in Table 4.2.

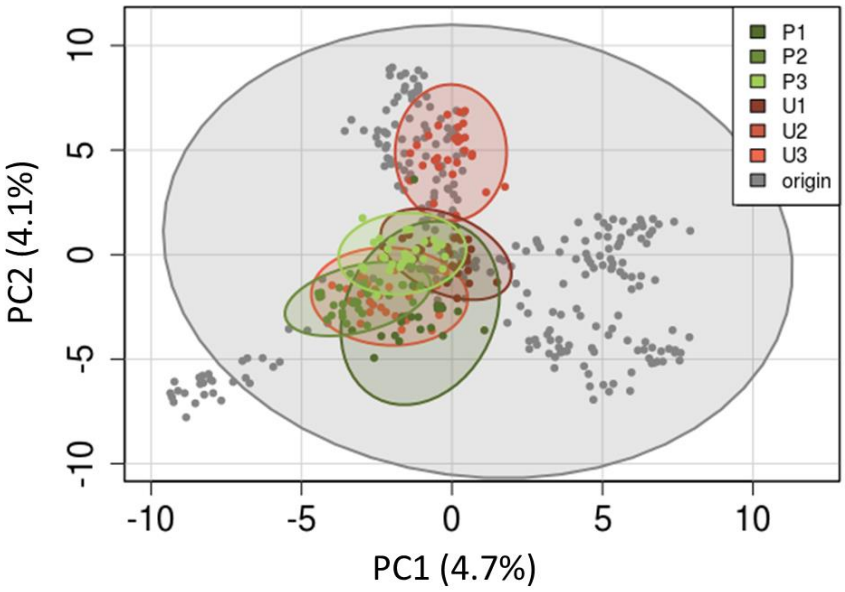


Figure 4.1. Principal component analysis (PCA) plot for the 6,107 SNPs of *Brachionus plicatilis* clones from the six laboratory populations and the origin population. Dots indicate the location of the genotype of each clone in the space defined by the first (PC1; 4.7 % variance explained) and second (PC2; 4.1 % variance explained) principal components. Ellipsoids are the 95% confidence interval the different populations. Color code: green, populations under predictable regime; red, populations under unpredictable regime; grey, origin population.

Table 4.2. Population pairwise fixation index (F_{ST}) values for the origin population and the six laboratory populations subjected to experimental evolution. P_i: populations evolved under the predictable selective regime; U_i: populations evolved under the unpredictable selective regime. The subindex *i* denotes replicate population within selective regime.

Population	P_1	P_2	P_3	U_1	U_2	U_3
P_2	0.025					
P_3	0.016	0.021				
U_1	0.038	0.038	0.036			
U_2	0.055	0.067	0.056	0.078		
U_3	0.035	0.034	0.035	0.046	0.071	
Origin	0.005	0.008	0.004	0.013	0.015	0.010

Observed heterozygosity (*Ho*), expected heterozygosity (*He*) and inbreeding coefficient (F_{IS}) for the origin and the six laboratory populations are shown in Table 4.3. Expected heterozygosity (*He*) –i.e., genetic diversity– was maintained in all laboratory populations (range between 0.17 to 0.21), except for one of the populations evolved under the unpredictable regime (U_2) which had the lowest genetic diversity. These values of genetic diversity were similar to those in the origin population (*He*= 0.21). Overall, all populations analyzed were broadly in Hardy-Weinberg equilibrium (*HWE*; Figure A.2).

Table 4.3. Population means of observed heterozygosity (*Ho*), expected heterozygosity (*He*), and inbreeding index (F_{IS}) for laboratory and origin populations. P*i*: populations under the predictable selective; U*i*: populations under the unpredictable selective regime. The subindex *i* denotes replicate population within selective regime.

Population	*Ho*	*He*	F_{IS}
P_1	0.22	0.21	-0.03
P_2	0.20	0.19	-0.04
P_3	0.22	0.20	-0.05
U_1	0.21	0.20	-0.04
U_2	0.19	0.17	-0.09
U_3	0.21	0.20	-0.03
Origin*	0.17	0.21	0.13

*The origin population was composed by clones from nine field populations. Here overall results as a single population are shown (full details for each of field populations in Franch-Gras et al. in review).

Candidate SNPs under selection

A total of 76 candidate SNPs under selection were identified using BayeScan among the three established groups: (1) origin population, (2) predictable populations, and (3) unpredictable populations (Figure 4.2). From these candidate SNPs, 61% (47 SNPs) were in linkage disequilibrium (LD > 0.7) and physically associated to other SNPs (between two and five SNPs), resulting in 45 genomic

regions showing evidence of being under selection. Three of the candidate SNPs under selection were identified in the comparison between both selective regimes (Figure 4.2; Figure A.3). These three unlinked candidate SNPs (S4644_2726, S9060_3689 and S78024_5745) had undergone strong changes in allele frequencies in populations under the unpredictable regime, diverging from the origin population (see Table 4.4), to the extent that two of them became fixed in the three population replicates subjected to the unpredictable regime.

Genome-wide association analysis

A genotype-phenotype association analysis using the subset of genotyped clones for which life history traits were available, revealed five SNPs highly correlated with these traits. Four SNPs in two scaffolds (S1772_184, S27547_4262, S27547_4267 and S27547_4271) were identified as candidates to be related to the hatching fraction (Figure 4.3A; Figure A.4), with R^2= 0.45 for the SNP S1772_184 and R^2=0.38 for the other three SNPs. The three SNPs in scaffold S27547 were found to be in linkage disequilibrium (R^2= 1). Genotypes associated to low and intermediate hatching fractions (11.5-67.2%) for both genomic regions were found in 19 clones. Just one candidate SNP (S5425_9210) was identified as associated to the timing of sex (Figure 4.3B; see Figure A.4) with R^2= 0.33.

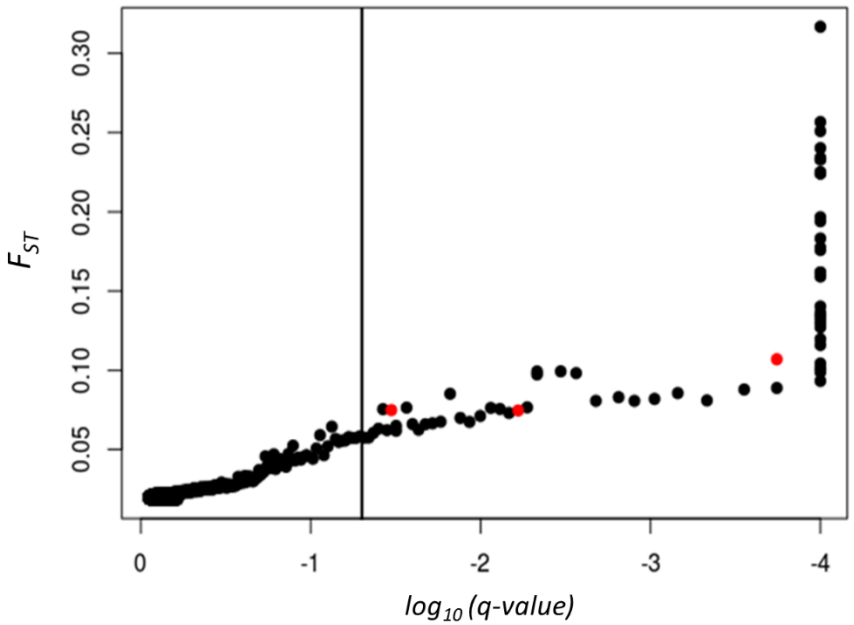


Figure 4.2. Identification of outlier loci putatively under selection in *Brachionus plicatilis* populations using BayeScan analysis for the 6,107 genotyped SNPs. The marker-specific F_{ST} is plotted against the decision factor to determine selection in base-10 log scale $\log_{10}(q\text{-value})$ using a false discovery rate (FDR) of 0.05. The vertical line is the critical prior odds (PO) of 10 used to identify outlier markers. Markers on the right side of the vertical line are outliers. This analysis included the origin population and the populations evolved under two selective regimes (predictable vs unpredictable). Each dot represents a SNP. Red dots indicate SNPs identified as being putatively under selection between selective regimes (see Figure A.3).

Table 4.4. Allelic frequencies in the origin and the six laboratory populations of *Brachionus plicatilis* for the three SNPs candidate to be under selection between both selective regimes. P*i*: populations under the predictable selective regime; U*i*: populations under the unpredictable selective regime. The subindex *i* denotes replicate population within selective regime.

Candidate SNP	Origin	P_1	P_2	P_3	U_1	U_2	U_3
S4644_2726	0.688	0.500	0.569	0.750	0.867	1.000	1.000
S9060_3689	0.924	0.914	0.724	0.857	1.000	1.000	1.000
S78024_5745	0.775	0.788	0.760	0.780	1.000	1.000	1.000

Identification of candidate genes under selection

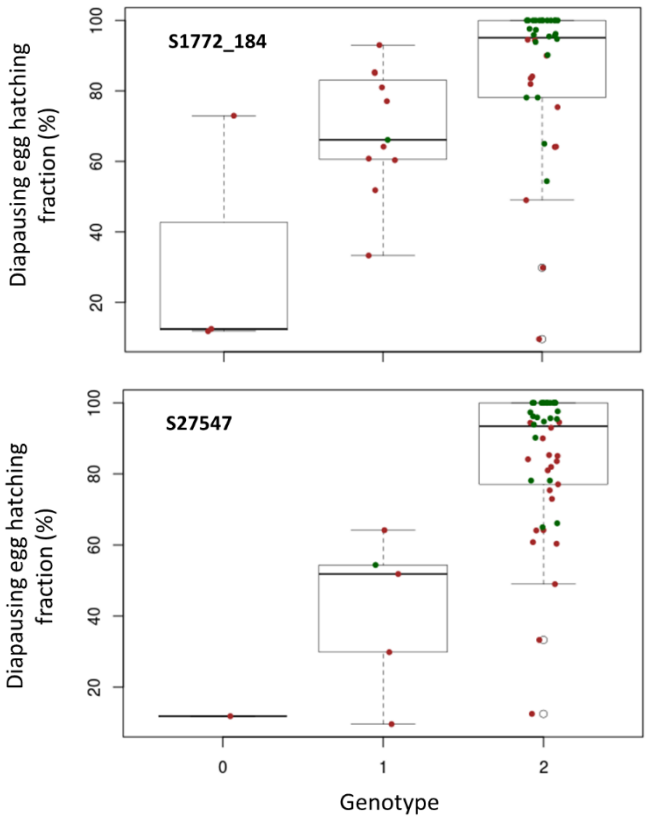
The 76 candidate SNPs identified by BayeScan to be under selection were located in 43 genes, 58.1% of which had putative functions associated (see Table A.2). Although these genes showed a wide range of functions, a GO enrichment analysis showed that they were enriched in the regulation of biological processes (GO:0050789), cellular processes (GO:0009987), single-organism processes (GO:0044699), regulation of cellular processes (GO:0050794), binding (GO:0005488) and biological regulation (GO:0065007). The three candidate SNPs to be under selection between selective regimes were located in three genes: *Ribosomal*

S6 kinase alpha-1-isoform X1, *RNA-binding single-stranded-interacting 3* and *Midasin*.

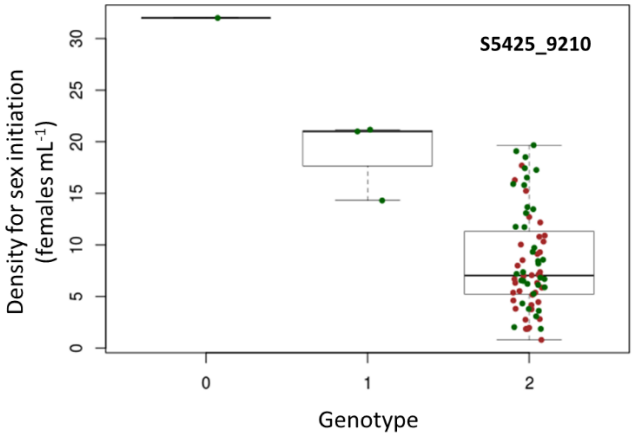
Those SNPs identified as putatively associated to the hatching fraction of diapausing eggs in the genotype-phenotype analysis were located in two genes, but only one had an associate function: the gene *kelch-like ECH-associated 1*. Additionally, no genes were found for the candidate SNP associated to the timing of sex.

Figure 4.3. Genotype-phenotype association patters for two life-history traits of *Brachionus plicatilis*: hatching fraction and timing of sex. Box-and-whisker plot of the genotypes of SNPs that are most significantly associated with (A) diapausing egg hatching fraction (SNP S1772_184 and three SNPs associated to the scaffold S27447), and (B) timing of sex, estimated as the density for sex initiation (SNP S5425_9210). The bottom of the box is the 25th percentile and the top is the 75th percentile, the median (straight line) is shown. The whiskers extend to the 10th and 90th percentiles. Dots represent each clone, those evolved under the predictable regime are represented by green dots, whereas clones evolved under the unpredictable one by red dots.

(A)


S1772_184
S27547
Diapausing egg hatching fraction (%)
Diapausing egg hatching fraction (%)
20
40
60
80
100
0
1
2
Genotype

(B)


S5425_9210
Density for sex initiation (females mL-1)
0
5
10
15
20
25
30
0
1
2
Genotype

Discussion

In this chapter, using an experimental evolution approach and genome-wide genotyping, genomic signatures of rapid adaptation to environmental unpredictability in rotifer populations under two contrasting laboratory selective regimes (predictable vs unpredictable) were identified using a GBS approach. Three out of 45 genomic regions putatively under divergent selection strongly shifted their allele frequencies in response to environmental unpredictability. Most of the remaining genomic regions experienced parallel allele changes in laboratory populations, suggesting adaptation to environmental laboratory conditions. As stated in Chapter 3, experimental laboratory populations showed signatures of adaptation to environmental unpredictability in their life-history traits (i.e., timing of sex and diapausing egg hatching fraction). Accordingly, the GWAS approach performed here revealed three genomic regions strongly associated to both life-history traits.

Laboratory populations were founded from nine highly genetically-structured *B. plicatilis* field populations that were obtained from Spanish Mediterranean salt ponds and lakes that cover a gradient of environmental predictability (see Chapter 2; Franch-Gras et al. 2017a; 2017b). Therefore, they were expected to contain high genetic variance underlying population adaptation to the degree of

predictability. Therefore and taking into account the relatively short-term scale of the experiment (Chapter 2), the adaptive evolution in the laboratory populations would result of selection on the standing genetic variation present in the founding populations rather than the rising of new genetic variants (Barrett and Schluter 2008; Hohenlohe et al. 2010; Stapley et al. 2010; Schlötterer et al. 2015). In support of this, candidate loci under selection were polymorphic in the founding populations (Table A.3) suggesting the existence of adaptive tracking in the populations evolved under the selective regimes (Tufto 2015). Moreover, this response may be facilitated by the existence of a diapausing egg banks in the experimental populations. These banks, used to restart the successive selective growing cycles in the experimental evolution design, would have acted as a reservoir of genetic diversity (Cousyn et al. 2001; Breendonk and De Meester 2003).

In the present work, three SNPs located in three different genes were detected as candidates to be under divergent selection in the unpredictable environments. Alleles in two of these SNPs (S9060_3689 and S78024_5745) became fixed in the three replicate populations evolving under the unpredictable regime. Such a consistent pattern of allele divergence between populations evolved under the two different selective regimes suggests that level of predictability may be acting as a selection factor on these candidate genomic regions (Hegreness and Kishony 2007; Orsini et

al. 2012; Burke et al. 2014). The SNP S9060_3689 was located within the gene *RNA-binding single-stranded-interacting 3* (RBMS3), a member of the family of genes c-myc single strand binding proteins (MSSP) which are involved in DNA replication, gene transcription, cell cycle progression and apoptosis in humans (Penkov et al. 2000), and that regulate one of the major pathways that promotes chondrogenesis in zebrafish (Jayasena and Bronner 2012). The SNP S4644_2726 was located within the *ribosomal S6 kinase alpha-1 isoform X1* gene, involved in controlling growth and differentiation in human cells (Moller et al. 1994). Finally, the SNP S78024_5745 was located in the *Midasin* gene, which codifies a nuclear chaperone involved in assembly/disassembly of complex macromolecules in yeast (Garbarino and Gibbons 2002), and has been shown to be essential for normal progression of female gametogenesis in *Arabidopsis thaliana* (Chantha et al. 2010). Despite the functions associated to these SNPs, further inferences cannot be drawn since the genetic data for *B. plicatilis* –a non-model species– is scarce and the draft genome used here has many genes with general functions. Regarding the genotype-phenotype association analysis, four putatively SNPs highly associated to diapausing egg hatching fraction (R^2> 0.38) were found. Three of them were fully linked and located within the same gene –*kelch-like ECH-associated 1*–, which is involved in responses to oxidative stress (Itoh et al. 1999; Dhakshinamoorthy and Jaiswal 2001; Hayes

and McMahon 2009). This gene has been described in several groups, including fish (Penglase et al. 2015), insects (Kelso et al. 2002), mammals (Wakabayashi et al. 2003), etc.

Laboratory populations subjected to both selective regimes showed a low level of genetic drift, evidenced by the low genome-wide pairwise F_{ST} found between laboratory populations (F_{ST}< 0.1; mean F_{ST} between selective regimes was 0.015), when compared to the original field populations (F_{ST}= 0.25 in Montero-Pau et al. 2017; F_{ST}= 0.18 in Franch-Gras et al. in review). In general, populations maintained their genetic diversity throughout the experiment, except from one of the populations that evolved under the unpredictable regime, in which a slight decrease in genetic diversity was observed. Nevertheless, the negligible loss of genetic diversity (*He*), the low values of F_{IS} and the little change in F_{ST} between populations suggest that genetic drift processes were not determinant in comparison to selection (Kaweki et al. 2012; Furlan et al. 2012; Matos et al. 2015; Tinnert et al. 2016).

A strong signal of adaptation to laboratory conditions during the selection experiment was found since a number of candidate SNPs under selection with parallel allelic frequency changes in the six laboratory populations –independently of their selective regime– were identified. In fact, the putatively selected alleles in laboratory populations of some candidate SNPs became fixed in 20 of the 76

candidate SNPs, indicating a strong selective pressure and suggesting a selective sweep (Nielsen et al. 2005). These results were not unexpected (Harshman and Hoffmann 2000; Mueller et al. 2005) given that (1) laboratory populations were derived from the field, and (2) laboratory conditions other than the selective regime were identical and constant across the selective regimes in the evolution experiment (e.g., temperature, salinity, food type and availability, light, length of dry phase, etc.). This contrasts with the different and highly variable conditions experienced by the field populations (Mueller et al. 2005) from which the origin population was derived (Franch-Gras et al. 2017b; Chapter 2). Selective regimes mimicked the extreme patterns of selection imposed by the environmental unpredictability to which *B. plicatilis* natural populations are exposed (Franch-Gras et al. 2017a). The experimental design used in this thesis focused on the unpredictability of the hydroperiod length as it is expected to exert a strong selection pressure in ecologically relevant traits of rotifer life-history such as the diapausing egg hatching fraction and the timing of sex (García-Roger et al. 2014; 2017; Franch-Gras et al. 2017b). However, unpredictability likely generated by other environmental factors (e.g., salinity, temperature, etc.) could be expected to create additional selective pressures in natural rotifer populations that were not explored here.

In this chapter, genomic signatures of rapid adaptation in laboratory populations through experimental evolution have been identified, supporting the previous results of rapid phenotypic divergence in rotifer populations, which evolved their life history traits divergently over a short time span (Chapter 3). Despite that other experimental evolution studies have shown adaptation to a range of selective pressures in rotifers (see review in Declerck and Papakostas 2017; Walczyńska et al. 2017) and other well-known cyclical parthenogens such as *Daphnia* (Geerts et al. 2015; Jansen et al. 2015), their genomic basis have been little explored. Genomic signatures of adaptation to environmental anthropogenic stressors were found in *D. magna*, using an experimental evolution approach (Orsini et al. 2012), however, the genomic basis of adaptation to environmental unpredictability is practically unknown. Available research includes only a recent study in rotifer field populations in which genomic signatures of local adaptation to unpredictable environments were identified (Franch-Gras et al. in review). Nonetheless, the candidate genomic regions that could be key in the adaptation to unpredictable environments are not shared between both studies. So, what is the genetic architecture underlying the life history trait responses? Are there just three genes involved? This is unlikely as most complex traits are driven by a very large number of genetic variants (Boyle et al. 2017). Therefore, it is likely that many genes across the genome that have

small effects would not be detected as significant outliers with the power of typical evolution experiments. Alternatively, selective pressures associated to habitat unpredictability in the field might differ from those in the experimental evolution approach performed here (Collins 2010), and this could explain the discrepancies found between the results reported by Franch-Gras et al. (in review) and the present study.

Finally, and given the recent availability of a *B. plicatilis* draft genome (Franch-Gras et al. in review), it was possible to call SNPs more confidently and to identify putative genes under selection. Interestingly, a tentative functionality has been assigned to most of the genes containing those SNPs (57.7%). Nevertheless, the lack of functional annotation in many of the genes of *B. plicatilis* precludes the understanding of the mechanisms under selection in this rotifer.

In conclusion, this chapter shows genomic evidence of rapid adaptation in experimental populations of the rotifer *B. plicatilis*, building on previous results for ecologically relevant traits. Candidate SNPs under selection to unpredictable environments were identified, some of them associated to key life-history traits in the life cycle of this rotifer species. Given that these responses allow monogonont rotifers to adapt to unpredictable environments, the results of this chapter can serve as a basis for future studies focused

on adaptation. In addition, rotifer populations showed strong genomic signals of adaptation to laboratory conditions. Furthermore, this study shows the potential of using monogonont rotifer populations to evaluate signatures of adaptation in short-term experiments. Nonetheless, a better understanding of genome and trancriptome of *B. plicatilis* is needed in order to elucidate the genomic basis of adaptation to unpredictable environments.

5

Comparative transcriptome analysis of diapausing eggs from rotifer populations subjected to predictable and unpredictable laboratory environments

Summary

To obtain a global view of the molecular mechanisms associated to diapause in the response to environmental unpredictability, a transcriptome analysis on experimental populations of the rotifer *Brachionus plicatilis* evolved under two contrasting patterns of environmental fluctuation (predictable vs unpredictable) was conducted. The main objectives of this research were to identify and quantify the expression of genes involved in both the maintenance of homeostasis during diapause in these rotifers, and in the exit from it. To do that, the experimental evolution approach described in Chapter 2 was combined with RNA sequencing technology. Diapausing eggs produced by laboratory populations evolved under each selective regime were collected in the last cycle

of selection (eighth growing cycle). Thereafter, the diapausing eggs from each population were divided in two groups to be indeed subjected to conditions either promoting or blocking hatching. Gene expression was analyzed by means of RNA-seq performed on the dipausing eggs from the different combinations of selective regime and diapause condition. A total of 3,068 differentially expressed genes were identified in rotifer diapausing eggs among combinations. The analyses performed in this chapter revealed that genes related to diapause maintenance and termination were differentially expressed between the diapausing eggs that were produced in the unpredictable regime with respect to those that were produced in the predictable regime. Thus, this study extends the knowledge of the complex molecular and cellular events that take place during diapause.

Introduction

Diapause is a form of adaptation to temporal heterogeneity characterized by temporal suspension of metabolic activity, arrested development, and increased stress resistance (Denlinger 2002; Reynolds and Hand 2009; see Chapter 1). This form of dormancy allows to overpass periods of unsuitable conditions and has been described in many organisms such as arthropods, nematodes and rotifers (Hand et al. 2016; Alekseev et al. 2012). In contrast with quiescence –immediate response to environmental conditions (Ricci 2001; Poelchau et al. 2013)– that is controlled exogenously, diapause depends on endogenous control for its initiation (Brendonck 1996). Thus, metabolic arrestment remains, even if suitable conditions resume, until specific cues disrupt diapause (Tauber et al. 1986; Alekseev et al. 2012).

Among rotifers, monogononts mostly produce diapausing forms as a by-product of sexual reproduction (but see Gilbert 1995; Chapter 2). These diapausing forms are encysted embryos called diapausing or resting eggs, so diapause in these rotifers is limited to the embryonic stage, in contrast to other organisms, such as insects, in which diapause can occur at any stage in their life cycle (Ricci and Pagani 1997; Gordon and Headrick 2001; Fan et al. 2013). Rotifer diapausing eggs are able to survive harsh environmental conditions such as drought, extreme temperatures and salinity fluctuations

(Ricci 2001; Schröder 2005; Hairston and Fox 2009), allowing population persistence in their local habitats and dispersion to other ones (Brendonck and De Meester 2003; Schröder 2005). Interestingly, the linkage between sexual reproduction and dormancy has been suggested to promote maintenance of sexual reproduction in these rotifers, since the production of diapausing eggs provides a major short-term ecological advantage to sex (Serra et al. 2004; Stelzer and Lehtonen 2016).

Diapause is considered to be a dynamic process that involves at least three different ecophysiological phases: (1) the entrance into diapause (or diapause initiation), (2) diapause maintenance, and (3) diapause termination (Kostál et al. 2006; Ragland et al. 2011). Although these phases have not been accurately described in rotifers, a similar ecophysiological division is expected to occur. Regarding diapause initiation, there are several evolutionary hypotheses in rotifers for the optimal timing of sex and diapausing egg production (for review see Serra et al. 2004; Gilbert 2012). Habitat deterioration, probability of male-female encounter, and resource demand have been hypothesized to drive the evolution of the timing of diapause, but so far empirical studies have not provided an unequivocal and definitive test to discriminate between these hypotheses. Nevertheless, there is general agreement that diapausing egg production may occur when resources are available for ensuring the storage of energetic

reserves that will be used for basal maintenance during both dormancy and the resumption of development at the end of diapause (Gilbert 2004; Gilbert and Schröder 2004). This is supported by several studies that suggest that diapausing eggs in some rotifer species are metabolically expensive and resource demanding, as evidenced by (1) demographic costs (i.e., lower fertility in diapausing-egg producing females compared to asexually-reproducing females; Gilbert 2004; Gabaldón and Carmona 2015), (2) sugar accumulation during diapause induction (Caprioli et al. 2004), or (3) the presence of other energy-rich molecules in diapausing eggs (e.g., lipid droplets; Gilbert 2004; Walsh et al. 2014; Tan et al. 2016b).

Once produced, diapausing eggs sink to the sediment and remain dormant for a time period –called obligatory period of diapause (Hagiwara et al. 1995)– in which embryos do not respond to environmental stimuli that typically promote hatching. The length of this period is variable both among and within species (Schröder 2005). Recent research has shown that the minimum period of obligate diapause in rotifers can be very short for some genotypes, lasting only a few days (Becks and Agrawal 2010; Martínez-Ruiz and García-Roger 2015; Stelzer 2017). However, little is known about the metabolism of rotifer embryos during this phase, although some studies in other diapausing organisms have showed evidence of respiration (e.g., reduced, but effective oxygen consumption; see

for review Podrabsky and Hand 2015), as well as other various endogenous changes (e.g., lipid degradation and synthesis of stress-tolerant proteins; Denlinger 2002; Reynolds and Hand 2009). In the case of rotifers, a handful of recent studies have developed transcriptome resources for the species *Brachionus plicatilis*, which has helped in the discovery of key genes for survival during diapause. These genes are related to processes maintaining the stability and the integrity of cell compartments and macromolecules during this metabolically-arrested stage of the rotifer life cycle (Denekamp et al. 2011; Clark et al. 2012). Gene families that have been shown to be key for diapause maintenance are described in Box 5.1.

After the obligatory period of diapause, and in response to specific hatching stimuli indicating the resumption of suitable environmental conditions, a fraction of diapausing eggs typically hatches and restarts the life cycle. Interestingly, the necessary conditions to induce hatching are variable between species and often vary among populations within the same species (Wyngaard 1988; García-Roger et al. 2008; Walczyńska and Serra 2014). The main factors described to induce hatching include light, temperature, and salinity (Pourriot and Snell 1983; Minkoff et al. 1983; Hawigara and Hino 1989). The complete reactivation of metabolism and development is achieved at the end of diapause (Kostàl 2006). Molecular mechanisms underlying the basis of

diapause termination are in general unknown, although a number of genes involved in embryonic development may be expressed (see Box 5.1). A study with rotifers has shown that some of the genes classified as involved in diapause maintenance in Box 5.1, also appear to be expressed in diapausing eggs during the first hours of exposure to hatching stimulus (Denekamp et al. 2011), so that they could be also implicated in diapause termination.

The degree of environmental unpredictability is expected to select differently on the various phases of diapause, although in the present study the focus was on the maintenance and termination of diapause. Theoretical models have proposed that leaving diapause may be a bet-hedging strategy (Cohen 1966; Seger and Brockmann 1987; Philippi and Seger 1989; Simons 2011) in which the optimal diapausing egg hatching fraction is related to the probability of facing favorable conditions when resuming development (for review see García-Roger et al. 2014). According to theory, lower hatching fractions are expected under unpredictable environments if compared to predictable ones. This ensures that if adverse growing seasons occur unexpectedly, some diapausing eggs will remain viable in the bank, which constitutes an ecological and evolutionary reservoir to restore future populations (Hairston 1996; Brendonck and De Meester 2003). Diapausing eggs from environments that differ in the degree of predictability are

expected to show different hatching phenotypes, which can translate into terms of differential gene expression.

In this chapter, RNA sequencing (RNA-Seq) was used to identify and analyze gene expression differences between diapausing eggs produced under combinations of (1) two contrasting laboratory selective regimes (predictable vs unpredictable), and (2) two different experimental diapause conditions (passing or not through a period of forced diapause in which hatching was inhibited). After such period of forced diapause, it is expected that a higher proportion of diapausing eggs will have overpassed the refractory period of obligate diapause, so that they would reactivate immediately under proper hatching conditions. Taking into account the divergent pattern found in the hatching fraction between selective regimes –higher hatching fractions under predictable regime (Chapter 3)–, it could be expected that a higher number of genes involved in diapause termination might be expressed under the predictable regime. In contrast, the lower hatching fractions in diapausing eggs produced under the unpredictable regime suggest that more eggs remain under metabolic and development arrestment. Therefore, to test this hypothesis, the analyses were mostly targeted at (1) the above-mentioned gene families, as they are key factors for survival during diapause, and (2) the study of the expression profile of genes related to leaving diapause or keeping in it.

Box 5.1. Gene-protein families associated to diapause with special emphasis on rotifers.

DIAPAUSE MAINTENANCE

Late embryogenesis abundant (LEA) proteins and trehalose metabolism. LEA proteins and trehalose are related to dormancy in many prokaryotes and eukaryotes, and known to be synthesized under dehydration conditions to stabilize other proteins and membranes during drying (Crowe et al. 2001; Wise and Tunnacliffe 2004; Hand et al. 2011a). Particularly in rotifers, LEA proteins have been found to maintain membrane integrity in bdelloids (Pouchkina-Stantcheva et al. 2007), and are associated to diapausing eggs in the monogonont *B. plicatilis* (Denekamp et al. 2009; 2010). On the other hand, trehalose is known to accumulate in diapausing cysts of *Artemia* (Clegg 1965; Moore and Hand 2016) and in rotifer diapausing eggs when they enter in anhydrobiosis (Caprioli et al. 2004; Denekamp et al. 2009). Trehalose acts as water replacement molecule and vitrifying agent, which serves to stabilize cell membranes (Tunnacliffe and Lapinski 2003). It is also associated to stress responses in nematodes (Pellerone et al. 2003).

Antioxidants. Oxidative stress leads to the accumulation of toxic components called reactive oxygen species (ROS). Therefore, the production of antioxidant enzymes are critical for stress resistance during diapause (Sim and Denlinger 2011) and has been shown in a wide range of organisms such as insects (Ragland et al. 2010), nematodes (Duceppe et al. 2017) and rotifers (Denekamp et al. 2009). The major ROS detoxifying enzymes described in rotifers include peroxidases, thioredoxins, catalases and glutathione-S-transferases (Denekamp et al. 2009; Clark et al. 2012).

Oxidoreductases. A number of proteins with oxidoreductase activity have been described to be involved during diapause with a role in detoxification processes and degradation of xenobiotics (Clark et al. 2012). For example, the *cytochrome P450 monooxygenase* gene has been shown to be overexpressed in insects during diapause (Reynolds and Hand 2009; Poelchau et al. 2013).

Box 5.1. (continued)

Heat Shock Proteins (HSPs). These proteins act as molecular chaperones by preventing abnormal protein folding during environmental stresses, such as: heat, cold, food depletion, osmotic stress, and toxicants (Van Straalen and Roelofs 2011; Feder and Hoffmann 1999; Liberek et al. 2008). In several insect species, HSPs are highly up-regulated upon entry into diapause (Denlinger et al. 2001; Rinehart et al. 2007; Li et al. 2007), and also involved in cell cycle arrestment (Denlinger 2002). In zooplankton, such as *Artemia* and rotifers, HSPs have been shown to be essential for thermotolerance (Clegg et al. 2001; Smith et al. 2012). However, several studies have shown that some HSPs can be up-regulated during diapause termination (Gkouvitsas et al. 2008; Reynolds and Hand 2009).

Aquaporins. These transmembrane proteins regulate the flow of water and/or small soluble molecules (e.g., glycerol) through the cellular channels (Kruse et al. 2006), allowing osmotic conditions to be maintained (Drake et al. 2015; Torson et al. 2017). During diapause, these proteins have been shown to be important in desiccation resistance in rotifers (Denekamp et al. 2009) and cold tolerance in insects (Philip et al. 2008; 2011; Lu et al. 2013).

Lipid and fatty acid metabolism. Lipid metabolism is considered to be important during diapause because lipids have been reported to be the principal source of energy during arrestment of embryonic development (Gilbert and Schröder 2004; García-Roger and Ortells 2018). Rotifer diapausing eggs contain abundant lipid droplets (Wurdak et al. 1978), so genes related to lipid metabolism could be involved in the maintenance of diapause (Alekseev et al. 2012; Pierson et al. 2013; García-Roger and Ortells 2018).

Defense response. During diapause, eggs are exposed not only to abiotic factors but also to biotic ones (i.e., fungi and bacteria). Thus, in addition to the surrounding egg layer (Wurdak et al. 1978) that protects them

Box 5.1. (continued)

against adverse environmental factors (Seidman and Larsen 1979; Couch et al. 2001; Alekseev et al. 2010), key components involved in innate immune response have been described during diapause. Toll-like receptors and F-box proteins have been shown up-regulated in rotifer *B. plicatilis* (Clarke et al. 2012), *Artemia* (Qiu et al. 2007) and insects (Roeder et al. 2004; Fan et al. 2013).

EMBRYONIC DEVELOPMENT

Lipid and fatty acid metabolism. Besides rotifer diapausing embryos, several studies have reported the presence of large numbers of lipid droplets in rotifer stem females (i.e., the females hatching from diapausing eggs), suggesting the importance of these stored lipids in post-hatching development (Gilbert 2004). In fact, Gilbert and Schröeder (2004) stated that lipid content in diapausing eggs might have evolved as a strategy to maximize the survival of stem females in either unpredictable or low food resource environments.

Cytoskeleton. Principal cytoskeletal components (filamentous, actin and tubulin) have important roles in a set of cellular functions such as cell division, motility and cell differentiation (Amos and Amos 1991; Herman 1993; McIlwain and Hoke 2005). Cytoskeleton changes have been related to early development in bdelloid rotifers (Boschetti et al. 2005) and zebrafish (Bonneau et al. 2011; Eno et al. 2016).

Morphological development, through the modification of the cytoskeleton or through other processes, is an important phase in embryo development after diapause. More specifically, several proteins such as ependymin, counting, notch, and innexins have been related to morphological development in rotifers (Clark et al. 2012).

Material and Methods

Diapausing egg collection

Diapausing eggs in which the comparative transcriptom analysis was performed were produced by *B. plicatilis* laboratory populations evolved in an experimental evolution design (see full details in Chapter 2).

Gene expression was analysed in diapausing eggs produced under two selective regimes of environmental fluctuation –(1) predictable (P) and (2) unpredictable (U). To do this, at the end of the last cycle of selection (eighth growing cycle), diapausing eggs were collected, cleaned up and counted. Diapausing eggs obtained for each laboratory population were divided into two groups to be subjected to two different diapause conditions: (1) "non-forced diapause" (NFD) and (2) "forced diapause" (FD). For the first condition, 5,200 newly produced diapausing eggs per laboratory population were placed in Petri dishes and immediately induced to hatch under standard hatching conditions (constant light, 6 g L^{-1} salinity and 25 °C; García-Roger et al. 2006) during 30-36 hours. This period of time is considered to be enough as to reactivate diapausing embryo development, but not to complete hatching (Denekamp et al. 2011; personal observation: no hatchlings were observed when handling the diapausing eggs). Thus, diapausing eggs were exposed to hatching conditions and not forced to remain in diapause (NFD). For

the second condition, the remaining 5,200 diapausing eggs per laboratory population were air-dried in darkness at 25 ºC, and afterwards stored in darkness at 4 ºC for 28 days. These conditions typically inhibit hatching (Pourriot and Snell 1983; García-Roger and Ortells 2018), so diapausing eggs were forced to remain in diapause (FD) during this period of time. After this period of forced diapause, diapausing eggs were placed in Petri dishes and induced to hatch under standard hatching conditions –as in NFD condition– during 30-36 hours. Therefore, a total of 12 diapausing egg samples resulting from 2 selective regimes (P vs U) x 2 diapause conditions (FD vs NFD) x 3 laboratory replicate populations were obtained.

Hatching experiment

A subsample of ca. 200 diapausing eggs from each sample was placed in 96-multiwell plates (Nunc™) and induced to hatch under constant light, 6 g L^{-1} salinity and 25 ºC (standard hatching conditions; García-Roger et al. 2006). These plates were monitored daily for 28 days in order to estimate diapausing egg hatching fraction and the timing of hatching.

RNA extraction

The remaining 5,000 diapausing eggs of each sample were subjected to two additional washes with DNase/RNase-free water (Gibco, Life Technologies). Once washed, the diapausing eggs of

each sample were placed in 1.5 mL microcentrifuge tubes and any remaining water was removed. Total RNA of each sample was isolated using TRIzol Plus RNA Purification kit (Invitrogen, Ambion, Life Technologies) following the manufacturer's instructions. In the first step of RNA isolation, once TRIzol® Reagent was added, a pellet pestle motor was used in order to homogenize the sample and assess the lysis of all diapausing eggs. DNase treatment during RNA purification was performed in order to reduce the amount of genomic DNA. At the end of the RNA extraction protocol, each sample was quantified and the 260/280 and 260/230 ratios were estimated with a NanoDrop spectrophotometer (Thermo Scientific). Since NFD condition was started one month earlier than FD condition, the total RNA isolated of NFD samples was kept at -80 °C. When the 12 samples of RNA were obtained, they were sent frozen to the *Servei Central de Suport a la Investigació Experimental* (SCSIE) of the University of Valencia (Spain) where RNA-seq was performed (see protocol in Chapter 2).

RNA-seq

In the Genomic core facility at SCSIE, the samples were quantified with Qubit 2.0 Fluorimeter (Life Technologies) and the purity and integrity of RNA were assessed through electrophoresis in Bioanalyzer 2100 (Agilent). After this quality control, RNA libraries were constructed using TruSeq stranded mRNA (Illumina) with an

enrichment of mRNA with standard poly-A based method followed by a chemical fragmentation and cDNA synthesis. RNA libraries were sequenced using 75 nt single-read sequencing on an Illumina NextSeq 500 platform. Quality of raw reads was assessed using FastQC version 0.11.5 (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapter sequences were trimmed and reads that had lengths shorter than 20 nucleotides were discarded.

Data analysis

Hatching data

The fraction of hatched eggs for each laboratory population and diapause condition assayed (NFD and FD) was estimated excluding the deteriorated eggs (García-Roger et al. 2005). Hatching fraction of diapausing eggs was analyzed using a generalized linear model (GLM) with binomial errors and logit as link function (Nelder and Wedderburn 1972). The timing of hatching was assessed by means of Kaplan–Meyer survival analysis on the cumulative hatching curves of the different populations. Censoring was applied to diapausing eggs that did not hatch by the end of the experiment. The non-parametric log-rank test was performed to test if there are differences in the timing of hatching among regimes and diapause conditions. Synchrony of diapausing egg hatching was estimated for each laboratory population by calculating the IQR/H_{50} index as a measure of dispersion (Bonett 2006), where IQR is the interquartile

range, and H_{50} is the median hatching time of hatched eggs (i.e., time in days needed for 50% of eggs to hatch). Thus, the higher the IQR/H_{50} index, the greater the hatching asynchrony. Differences between selective regimes (P vs U) and between diapause conditions (FD vs NFD) in the IQR/H_{50} index were assessed using ANOVA. All analyses were performed in R, version 3.2.2 (R Development Core Team 2015). GLM and ANOVA were respectively implemented by means of the *glm* and *lm* functions (R Development Core Team 2015). For Kaplan-Meyer survival analysis, the *survdiff* function from the "survival" package (Therneau 2015) was used.

Transcriptome assembly

Transcriptome assembly was performed using the Tuxedo protocol (Trapnell et al. 2012). RNA-Seq reads were mapped to a draft genome of *B. plicatilis* (Franch-Gras et al. in review) *via Tophat* v. 2.1.1 (Kim et al. 2013) using the following parameters: -I/--max-intron-length= 15,000; -i/--min-intron-length= 10; --max-multihits= 1; --read-gap-length= 1; --read-realign-edit-dist= 0. For the rest of parameters default values were used. In order to assemble the aligned reads into transcripts *Cufflinks* software (Trapnell et al. 2012) was used modifying the following parameters: -N/--upper-quartile-norm; -q; -g/--GTF-guide; -I/--max-intron-length= 15,000, --min-intron-length= 10. Maximum and minimum intron length in *Tophat* and *Cufflinks* was assessed with the reference genome of *B.*

plicatilis. Furthermore, *Cuffmerge* –which merges assemblies from all samples– and *Cuffcompare* –which filters transcribed fragments that are likely to be artifacts– were used to produce a general assembly suitable for use in the next step. In all the cases, the values applied in the above mentioned parameters intended to follow a conservative approach to detect differential gene expression.

Differentially expressed genes (DEGs)

The following comparisons among combinations of selective regimes (P and U) and diapause conditions (NFD and FD) were explored: (1) U-NFD vs P-NFD; (2) U-FD vs P-FD; (3) U-NFD vs U-FD; and (4) P-NFD vs P-FD. Differential expression was determined on the three population replicates of each combination by using *Cuffdiff* –a software implemented in *Cufflinks*– with the following parameters: --b/--frag-bias-correct; -u/--multi-read-correct. Then, reads per kilobase of transcript per million mapped reads (RPKM) of each gene was calculated based on the length of the gene and reads count mapped to this gene (Trapnell et al. 2010). RPKM, also known as FPKM in pair-ended sequencing experiments (Trapnell et al. 2012), is the normalized measure to quantify differential expression. Statistical significance was determined based on the false discovery rate (FDR) adjusted *P*-values≤ 0.05 (Benjamini and Hochberg 1995). Those significant differentially expressed genes (DEGs) in which no expression was found in one sample for each

comparison (RPKM= 0), resulted in an infinite ratio in log_2-fold change (log_2-FC). In order to visualize the results for differential expression, CummeRbund v. 2.18.0 and ggplot2 *v*. 2.2.1 packages, both implemented in R version 3.2.2 (R Development Core Team 2015), were used. To adopt a conservative criteria, genes were considered to be differentially expressed between both selective regimes at each diapause condition when log_2-FC≥ 2 and *q*-value≤ 0.05; downstream analyses were performed with this set of DEGs.

Functionality assignment of DEGs and Gene Ontology (GO)

Gene functions were retrieved from the current annotation of the *B. plicatilis* genome (Franch-Gras et al. in review). In order to find functions related to diapause maintenance and embryonic development (i.e., diapause termination), genes of interest were manually investigated by using a custom *grep* script in bash (Unix Shell) with keywords (see Table A.3). DEGs in each comparison – those described in the above section– composed the test set to find enrichment Gene Ontology (GO) term using Fisher's exact test (FDR≤ 0.05, two-tailed analysis) in Blast2GO (Conesa et al. 2005). In order to remove those enriched GO terms that are too general from the results, a reduction to most specific at FDR≤ 0.01 was used.

Results

Diapausing egg hatching experiment

Both the selective regime and the diapause condition affected the diapausing egg hatching fraction; the effects of both factors and their interaction were significant (Table 5.1; GLM, *P*-values< 0.01). Hatching fractions were significantly lower under the unpredictable regime for both diapause conditions (Figure 5.1). Timing of hatching was also significantly affected by the selective regime (survival log-rank test; χ^2= 236, *P*-value< 0.001) and the diapause condition (χ^2= 28.1, *P*-value< 0.001).

Table 5.1. Summary of the general linear model (GLM) on diapausing egg hatching fraction and ANOVA for the IQR/H_{50} ratio. Degrees of freedom were 1 in all combinations.

	Hatching fraction (%)		**IQR/H_{50}**	
Effect	χ^2	***P*-value**	***F***	***P*-value**
Regime (P/U)	50.117	< 0.01	9.907	0.01
Condition (NFD/FD)	115.726	< 0.01	0.318	0.59
Regime (P/U) x Condition (NFD/FD)	7.445	< 0.01	0.012	0.91

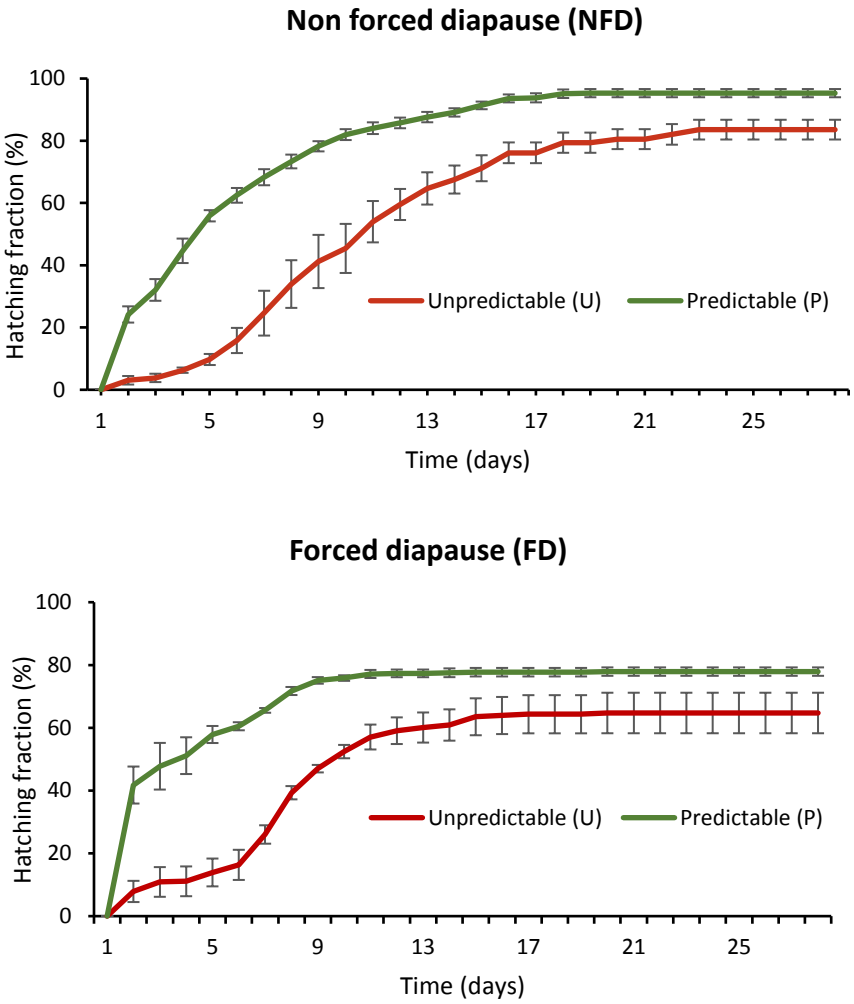


Figure 5.1. Cumulative percent hatching of *Brachionus plicatilis* diapausing eggs obtained under two laboratory selective regimes (predictable and unpredictable) after 28 days of incubation in two diapause conditions (non-forced and forced).

The relationship between diapausing egg hatching fraction and H_{50} showed a delayed hatching pattern in the populations from the unpredictable regime; H_{50} averaging 9.66 ± 1.20 days and 8.00 ± 0.57 days under NFD and FD conditions, respectively. Under the predictable regime H_{50} values averaged 4.66 ± 0.33 days, and 2.66 ± 0.66 days, respectively for NFD and FD conditions. No matter the selective regime, FD condition resulted in lower IQR/H_{50} values than NFD condition, and whatever the experimental condition, IQR/H_{50} was always higher in the predictable regime than in the unpredictable one (Figure 5.2).

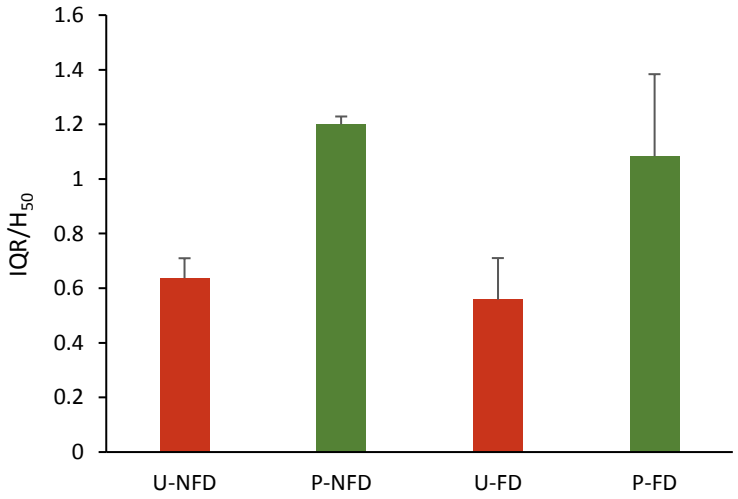


Figure 5.2. Asynchrony of diapausing egg hatching in *Brachionus plicatilis* as estimated by the IQR/H_{50} index in each combination of selective regime and diapause condition. Bars indicate ± 1SE.

Mapping and alignment of RNA-seq reads to *B. plicatilis* genome

High-throughput sequencing generated 24.8 Gb of raw reads. After a quality filter, for NFD condition, 50.18 ± 5.40 and 57.8 ± 1.07 million of reads were found under the predictable and unpredictable regimes, respectively. The number of reads obtained for FD condition were 35.05 ± 2.93 and 41.28 ± 2.32 million reads under the unpredictable and the predictable regime, respectively. Thus, the number of reads in FD was lower than in NFD. In both NFD and FD conditions, the mapped reads with the reference *B. plicatilis* genome were similar, 85% and 87% of mapped reads, respectively. Population values for each combination of selective regime and diapause condition are summarized in Table 5.2. An average of 36,896 ± 388 expressed genes in each key comparison were identified. The abundance of gene transcripts was expressed as RPKM (Trapnell et al. 2010). Quality control plot (Goff et al. 2013) of RPKM density distribution (i.e., the squared coefficient of variation (CV^2) vs $\log_{10}$FPKM) for each selective regime and diapause condition (U-NFD; U-FD; P-NFD; P-FD) is shown in Figure A.5.

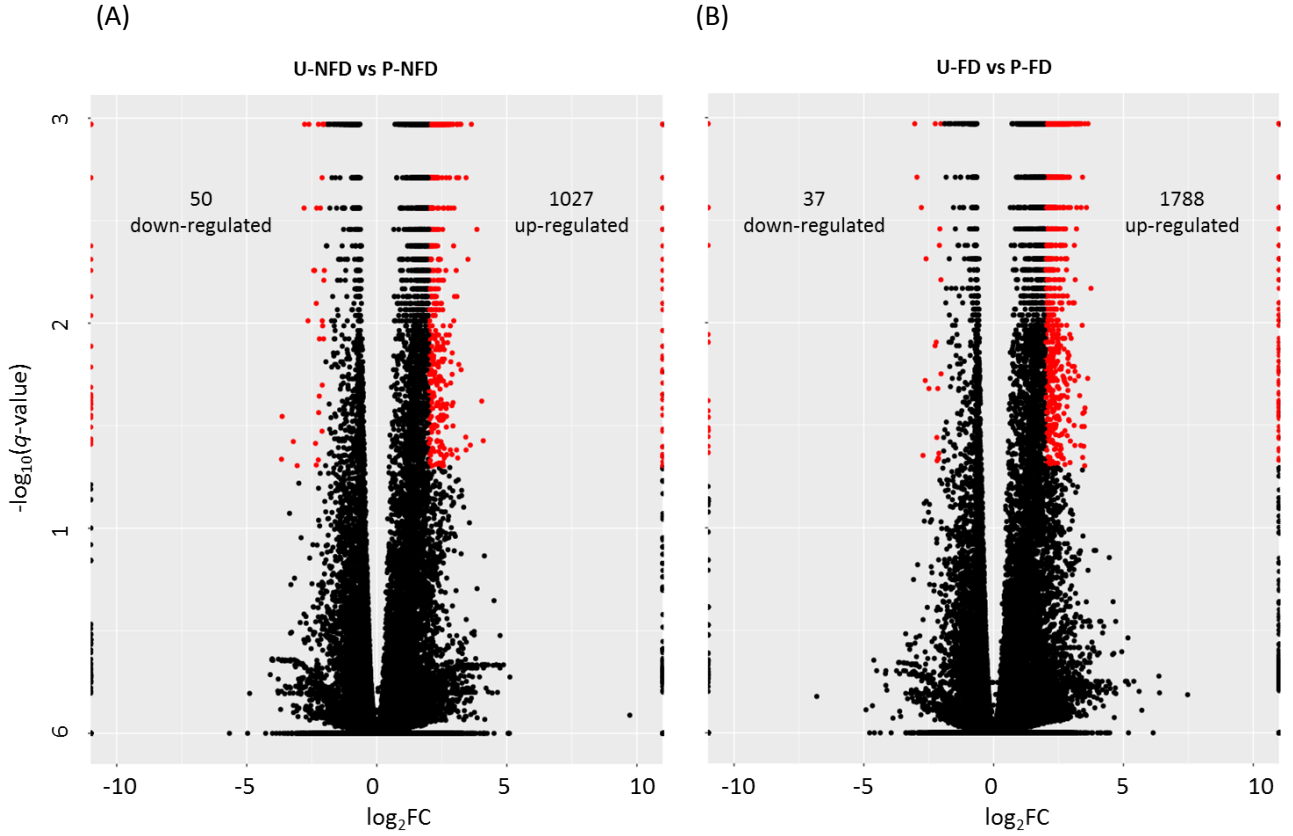
Table 5.2. Statistics for the filtering and mapping RNA-Seq reads corresponding to the combinations of selective regimes and diapause conditions. P: predictable regime; U: unpredictable regime; NFD: non-forced diapause; FD: forced diapause. Replicate corresponds to each one of the six experimental evolution populations.

Regime/ Condition	Replicate	Reads in	Reads mapped
U-NFD	1	58,364,853	46,103,494 (79.0%)
	2	55,711,941	49,369,286 (88.6%)
	3	59,268,714	51,793,879 (87.4%)
P-NFD	1	55,778,100	48,926,714 (87.7%)
	2	39,381,051	30,637,627 (77.8%)
	3	55,388,301	48,681,614 (87.7%)
U-FD	1	29,222,289	25,433,737 (87.0%)
	2	38,563,452	34,283,803 (88.9%)
	3	37,364,770	32,119,977 (86.0%)
P-FD	1	44,386,767	38,807,814 (87.4%)
	2	36,745,427	31,601,100 (86.0%)
	3	42,730,345	37,107,511 (86.8%)

Analysis of differentially expressed genes (DEGs)

Volcano plots of genes that were differentially expressed in the four comparisons illustrate distinct transcriptional profiles (Figure 5.3). A total of 3,068 significantly DEGs were found across the four key comparisons. Higher numbers of up-regulated DEGs were found in the predictable regime (1,027 and 1,788 DEGs under NFD and FD conditions, respectively) than in the unpredictable one (50 and 37 DEGs for NFD and FD conditions, respectively). Comparisons between diapause conditions within the same selective regime showed lower numbers of DEGs (100 and 70 DEGs, respectively for the U-NFD vs U-FD and P-NFD vs P-FD comparisons).

Figure 5.3. Volcano plots (statistical significance vs fold change (log_2-FC)) showing differential gene expression between selective regimes (P vs U) and diapause conditions (non- forced diapause, NFD vs forced diapause, FD). (A) U-NFD vs P-NFD; (B) U-FD vs P-FD; (C) U-NFD vs U-FD; and (D) P-NFD vs P-FD. Genes whose expression is significantly differentiated (*q*-value≤ 0.05) and showing a log_2-FC≥ 2 are in red. Not significantly differentiated genes are in black. Down- and up-regulated refer to the second item in each comparison.


(A)
U-NFD vs P-NFD
50
down-regulated
1027
up-regulated
-log10(q-value)
3
2
1
6
-10
-5
0
5
10
log2FC
(B)
U-FD vs P-FD
37
down-regulated
1788
up-regulated
-10
-5
0
5
10
log2FC

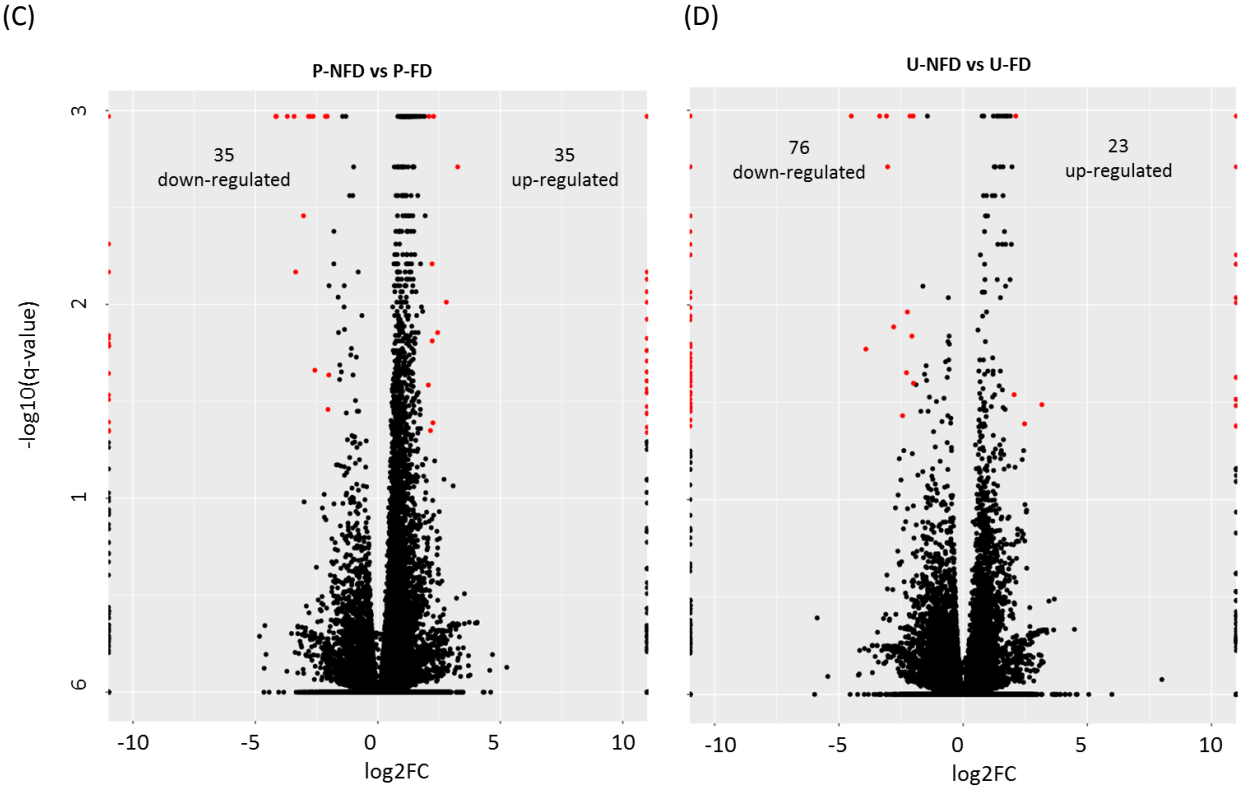


Figure 5.3. (continued)

The figure 5.4A shows the number of specific and shared DEGs in the four key comparisons (U-NFD vs P-NFD, U-FD vs P-FD, U-NFD vs U-FD, and P-NFD vs P-FD). Of the 568 DEGs found in both the U-NFD vs P-NFD and U-FD vs P-FD comparisons, 560 were found to be up-regulated in the diapausing eggs produced under the predictable regime in both comparisons, suggesting that the differential expression of those genes is related to the selective regime, independently of the diapause condition assayed (Figure 5.4B). Only eight genes were found to be differentially expressed in both the P-NFD vs P-FD and U-NFD vs U-FD comparisons, all of them being up-regulated in NFD. This result strongly suggests that diapause condition is not so important in driving differences in the transcriptional profile of these genes as it is the selective regime (Figure 5.4C).

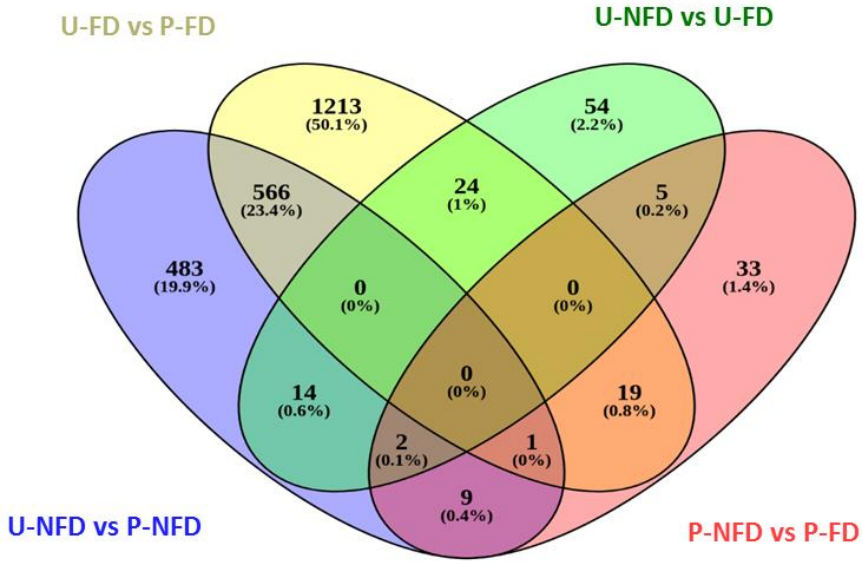
Gene function assignment of DEGs

After visual checking for functionality of DEGs in the four key comparisons, we found DEGs belonging to gene families related to diapause maintenance and embryonic development. Large numbers of gene families and high percentages of genes from each family were found to be differentially expressed in the between-regime comparisons (U-NFD vs P-NFD and U-FD vs P-FD; see Figure 5.5). By contrast, the number of DEGs was much lower in the within-

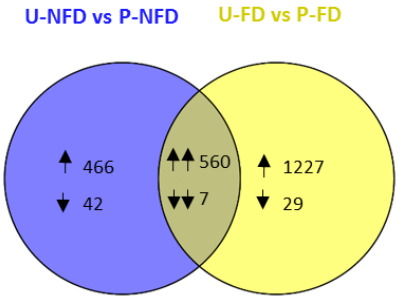
regime comparisons (U-NFD vs U-FD and P-NFD vs P-FD). Only five DEGs with functionality assignment were found in the U-NFD vs U-FD comparison (*fatty acid synthase, trehalase, Zinc metalloase-4-like, RNA-directed DNA polymerase,* and *adhesin*) and four in the P-NFD vs P-FD comparisons (*fatty acid synthase, C-type lectin domain, hypothetical protein GLOINDRAFT,* and *adhesin*) (data not shown). A broad range of gene ontology was found in U-NFD vs P-NFD and U-FD vs P-FD comparisons (Figure A.6), but no significant gene enrichment was found in the two other comparisons (U-NFD vs U-FD and P-NFD vs P-FD).

Figure 5.4. Venn diagrams showing: (A) The number of significant differential expression genes (DEGs) applying criteria of $-2 > \log_2\text{-FC} \geq 2$ and *q*-value≤ 0.05, for each comparison of selective regime and diapause condition (U-NFD vs P-NFD, U-FD vs P-FD, U-NFD vs U-FD, and P-NFD vs P-FD); (B) between-regime comparisons (U-NFD vs P-NFD and U-FD vs P-FD), and (C) within-regime comparisons (U-NFD vs U-FD and P-NFD vs P-FD). Up and down arrows represent the up- and down- regulated genes, respectively. P: predictable regime; U: unpredictable regime; NFD: non-forced diapause; FD: forced diapause.

(A)


U-FD vs P-FD
U-NFD vs U-FD
1213
(50.1%)
54
(2.2%)
566
(23.4%)
24
(1%)
5
(0.2%)
483
(19.9%)
0
(0%)
0
(0%)
33
(1.4%)
0
(0%)
14
(0.6%)
19
(0.8%)
2
(0.1%)
1
(0%)
9
(0.4%)
U-NFD vs P-NFD
P-NFD vs P-FD

(B)


U-NFD vs P-NFD
U-FD vs P-FD
466
42
560
7
1227
29

(C)

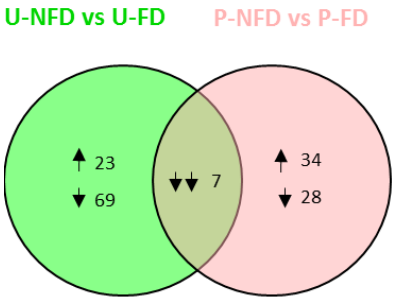
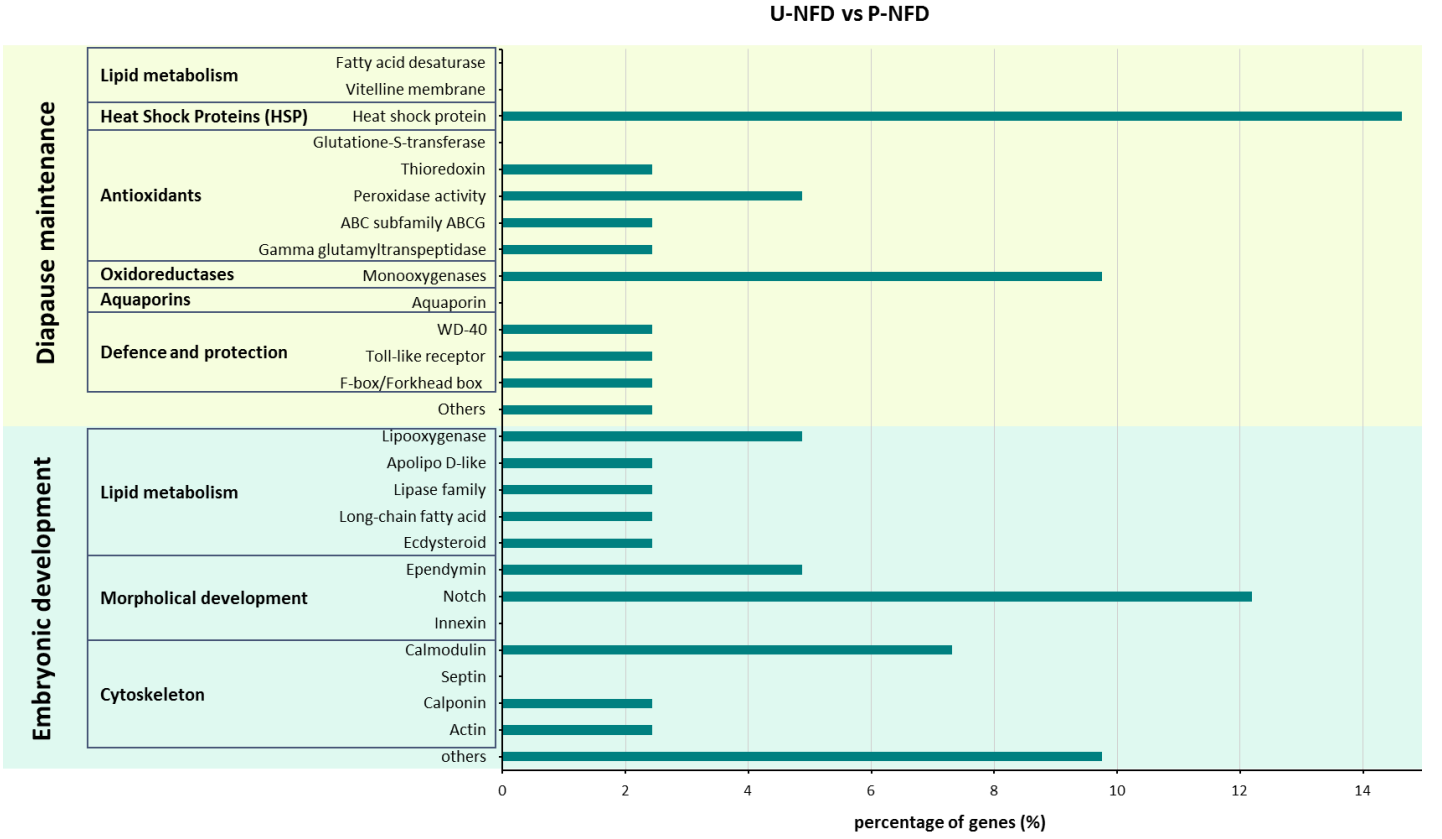
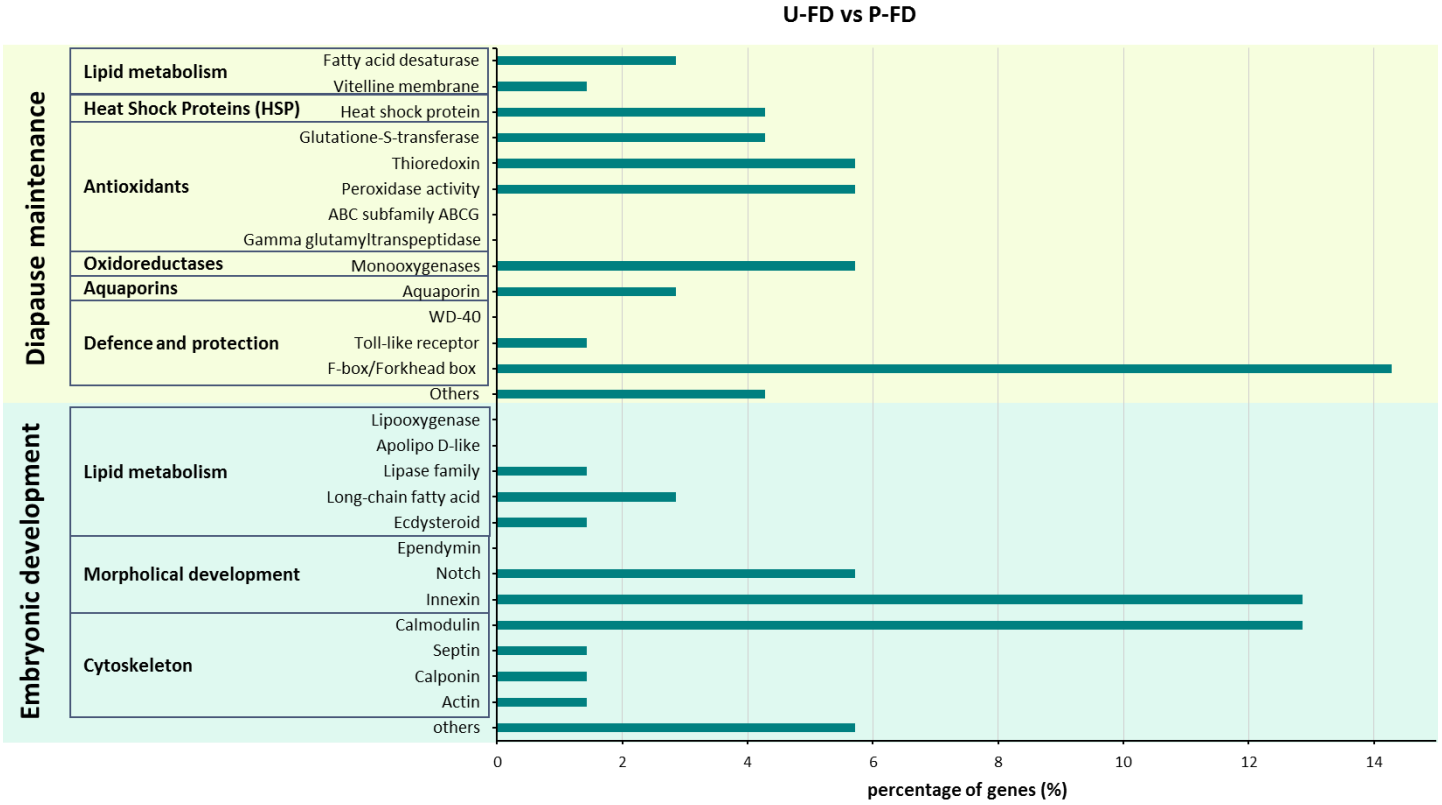

U-NFD vs U-FD
P-NFD vs P-FD
23
69
7
34
28

Figure 5.5. Percentage of differentially expressed genes (DEGs) between selective regimes in a set of families of genes that are related to diapause maintenance and embryonic development. The comparisons shown here are U-NFD vs P-NFD and U-FD vs P-FD in *Brachionus plicatilis* diapausing eggs. All DEGs were up-regulated in the predictable regime in both diapause conditions. P: predictable regime; U: unpredictable regime; NFD: non-forced diapause; FD: forced diapause.


U-NFD vs P-NFD
Diapause maintenance
Embryonic development
Lipid metabolism
Fatty acid desaturase
Vitelline membrane
Heat Shock Proteins (HSP)
Heat shock protein
Antioxidants
Glutatione-S-transferase
Thioredoxin
Peroxidase activity
ABC subfamily ABCG
Gamma glutamyltranspeptidase
Oxidoreductases
Monooxygenases
Aquaporins
Aquaporin
Defence and protection
WD-40
Toll-like receptor
F-box/Forkhead box
Others
Lipid metabolism
Lipooxygenase
Apolipo D-like
Lipase family
Long-chain fatty acid
Ecdysteroid
Morpholical development
Ependymin
Notch
Innexin
Cytoskeleton
Calmodulin
Septin
Calponin
Actin
others
0
2
4
6
8
10
12
14
percentage of genes (%)


U-FD vs P-FD
Diapause maintenance
Embryonic development
Lipid metabolism
Fatty acid desaturase
Vitelline membrane
Heat Shock Proteins (HSP)
Heat shock protein
Antioxidants
Glutatione-S-transferase
Thioredoxin
Peroxidase activity
ABC subfamily ABCG
Gamma glutamyltranspeptidase
Oxidoreductases
Monooxygenases
Aquaporins
Aquaporin
Defence and protection
WD-40
Toll-like receptor
F-box/Forkhead box
Others
Lipooxygenase
Apolipo D-like
Lipase family
Long-chain fatty acid
Ecdysteroid
Morpholical development
Ependymin
Notch
Innexin
Cytoskeleton
Calmodulin
Septin
Calponin
Actin
others
0
2
4
6
8
10
12
14
percentage of genes (%)

Discussion

This chapter focuses on a very relevant stage of the life cycle of *B. plicatilis*, the diapausing egg stage, and explores the patterns of diapause maintenance and termination. The study combined hatching experiments and transcriptomics on diapausing eggs produced by populations evolving under two divergent regimes of environmental predictability (see Chapter 2 for details), which were indeed subjected to conditions either promoting or blocking hatching. Different hatching patterns were observed among selective regimes and diapause conditions, and more than 3,000 genes with different expression profiles between the studied comparisons were identified. To the best of the author's knowledge, this is the first study that uses RNA-seq and a reference genome to relate hatching patterns with transcriptional profiles in diapausing eggs subjected to predictable and unpredictable laboratory environments. However, the present study benefits from other previous studies that have provided very useful genetic resources on the discovery and function assignment of genes associated to diapause and embryonic development in *B. plicatilis* (Denekamp et al. 2009; 2011; Clark et al. 2012), *Brachionus calyciflorus* (Hanson et al. 2013), and *Brachionus manjavacas* (Kim et al. 2015). This information is used below to establish links

between the observed hatching phenotypes and their corresponding RNA expression profiles.

In agreement to the well-established predictions derived from bet-hedging theory for rotifer diapausing egg hatching (García-Roger et al. 2014; Chapter 1 and 3), lower hatching fractions in the diapausing eggs produced by populations subjected to the unpredictable regime were observed –regardless of the two diapause conditions assayed (NFD and FD)– if compared to the predictable one. Variable diapause duration and intermediate hatching fractions have been proposed as adaptations to environmental unpredictability in rotifers (Schröder 2005; García-Roger et al. 2017; Chapter 3). Diapausing eggs from the predictable regime showed higher, earlier and a more asynchronous hatching than those from the unpredictable regime. Interestingly, these traits have been observed to quickly evolve under the divergent predictability regimes assayed in the experimental evolution approach used here (Chapter 3). Also consistent with the initial hypothesis, the timing of diapausing egg hatching was earlier and more synchronous after a forced period of diapause. A striking result was that hatching fractions were slightly lower in the FD than in the NFD condition, which could be explained by an increased diapausing egg mortality due to the forced diapause period (Cáceres and Schwalbach 2001; De Stasio 2012), despite that all the eggs in the assays looked healthy (García-Roger et al. 2006).

Out of the 3,068 DEGs identified across the four key comparisons, 2,900 DEGs were found in the comparisons between the two contrasting selective regimes for both diapause conditions (U-NFD vs P-NFD and U-FD vs P-FD comparisons), of which 25% were shared between both comparisons. This result suggests that the selective regime is more important in driving differences in the transcriptome profile of diapausing eggs than the diapause condition assayed. This relatively low relevance of diapause conditions on gene expression has been also evidenced in a recent study where conditions were similar to those used in the present experiment (Ziv et al. 2017). Interestingly, most of the DEGs were up-regulated in the predictable regime, whatever the diapause condition (95.3% in NFD, and 98.0% in FD). It is likely that some DEGs overexpressed in the predictable regime could be related to the reactivation of diapausing eggs and hatching readiness. This would be in concordance with the results from the hatching experiment in which diapausing eggs produced under the predictable regime showed earlier and higher hatching. In fact, a family of genes related to morphological development and cytoskeleton (i.e., calmodulins, calponins, innexins, actins, ependymins...) were up-regulated in the predictable regime in both diapause conditions. Previous studies have reported the expression of several genes associated to light stimulation and embryonic development after a short exposure to illumination (Denekamp et

al. 2011; Kim et al. 2015). Consistently, two DEGs (*rhodopsin* and *photoreceptor-specific nuclear receptor-like)* were found to be up-regulated in P-NFD with respect to U-NFD, and the *melanopsin, rhodopsin* and *arthropsin* genes were found to be up-regulated under P-FD condition with respect to U-FD condition. Other gene families involved in photoreception (e.g., opsins, haem pigments, cryptochromes) that could be involved in the response to light stimulus for leaving diapause (Kashiyama et al. 2010; Vanvlasselaer and De Meester 2010; Pinceel et al. 2013; Kim et al. 2015) were also found in the present study although that the differential expression between the experimental comparisons were not significant. On the other hand, a lower, more synchronous and delayed hatching was found under the unpredictable regime in both diapause conditions if compared to the predictable regime. This hatching pattern is consistent with the general down-regulation pattern observed in the U-NFD and the U-FD with respect to the P-UNF and the P-FD, which could be associated to the developmental arrest during diapause (Alekseev et al. 2012; Fan et al. 2013; Kôstal et al. 2017).

Rotifer embryos are able to survive very harsh conditions during diapause (Hairston and Fox 2009; Radzikowski 2013), which requires both metabolic re-structuring and stress tolerance (Hand et al. 2011b; Poelchau et al. 2013; Podrabsky and Hand 2015). Not surprisingly, evidence for expression of genes related to thermal

and oxidative stress was found in the diapausing eggs for all the combinations between selective regime and diapause condition. Noticeably, more than 20% out of the DEGs found to be related to diapause were associated to antioxidants and oxidoreductases. This result is aligned with previous studies showing that the production of antioxidant enzymes is critical for stress resistance during diapause (Sim and Denlinger 2011). Consistent with previous reports on rotifer diapausing eggs, we found evidence for expression of thioredoxins, peroxidases, and Glutathione-S-transferases (Denekamp et al. 2011; Clark et al. 2012). Genes related to the oxidoreductase activity, such as *cytochrome P450*, were found in the comparisons between selective regimes in both diapause conditions. Moreover, as well as genes involved in defense and protection, such as C-type lectin receptors and Toll-like receptors. These receptors are components of the innate immune system related to antifungal defense (Willment and Brown 2008; LeibundGut-Landmann et al. 2012; Hao et al. 2012). In fact, C-type lectin receptors have been related to the response to stimulus, as it could be the response to hatching cues. Also of special interest, DEGs related to aquaporins were only found under FD conditions, no matter the predictability regime. This could be related to the fact that these proteins are involved in protection to desiccation (Philip et al. 2008; Cohen et al. 2012). Thus, results are in agreement with desiccation being only experienced by diapausing eggs under the

FD conditions, whereas under the NFD condition diapausing eggs were always hydrated in the experimental design.

Gene assignment of function to either diapause maintenance or embryonic development processes, although based on previous literature, is still tentative and further research is needed. In fact, several studies have shown that gene families traditionally associated to diapause maintenance –such as some glutathione-S-transferases, aquaporins, ferritins, or trehalose metabolism genes (Denekamp et al. 2009)– are highly expressed in diapausing eggs after exposure to 30 hours of light (Denekamp et al. 2011), so that these genes could be also involved in embryonic development reactivation. In agreement with this, some of these gene families (e.g., glutathione-S-transferases and aquaporins) were more expressed in the diapausing eggs from the predictable regime than in the ones from the unpredictable regime. Notice that given the higher hatching fractions of diapausing eggs produced under the predictable regime, a higher number of genes involved in diapause termination is expected to be expressed in these eggs when induced to hatch. In fact, after 30 hours of exposure to hatching conditions, embryo movement was observed in many of the eggs from the predictable regime (personal observation), supporting that these genes could be involved in embryo reactivation. Some other genes or gene families may have an ambivalent role in rotifer diapause, as it could be the case of genes related to lipid

metabolism (Box 5.1). Interestingly, the *fatty acid synthase* gene was found to be up-regulated under NFD conditions in both selective regimes. The enzyme encoded by this gene has been shown to be involved in lipid accumulation and stress tolerance during diapause (Tan et al. 2016a). A differential expression in a gene codifying a vitelline membrane protein (*vitelline-membrane outer layer)* was found between selective regimes under the FD condition, this gene being up-regulated under the predictable regime. The major role of this membrane is to avoid mixing of yolk, although it also acts as an anti-microbial barrier (Sricharoen et al. 2005). This gene has also been found up-regulated in post diapause offspring of *Daphnia* (Kaupinis et al. 2017). Harvesting lipids for the yolk should start at the timing of diapausing egg production (i.e., diapause initiation). However, the timing of mobilization of yolk resources is not clear yet. A hypothesis to be tested is whether the usage of these lipids could be arrested during diapause and resumed just right at the timing of reactivation by using the stored lipids that have not been depleted during diapause. This makes sense since large numbers of droplets with neutral lipids have been found in females that hatch from diapausing eggs (Gilbert 2004). This finding supports the importance of lipids in rotifer development after hatching and in the improvement of their ability to colonize new environments (Alekseev et al. 2006; Gilbert 2012).

Despite the recognition of the importance of LEA proteins and trehalose during diapause in several investigations (Tunnacliffe and Wise 2007; Hand et al. 2011a), no differential expression in the genes associated to them was found in the present study. This could be because their expression is highly conserved in diapausing eggs, independently of the selective regime and conditions during diapause. In fact, some genes involved in trehalose synthesis showed a different level of expression between selective regimes, but their log_2-FC values were lower than the conservative threshold value established. The *trehalase* gene was found to be differentially expressed between diapause conditions in the unpredictable regime. The enzyme encoded by that gene, is known to be involved in the conversion of trehalose to glucose, which is used as an energy source during diapause (Duceppe et al. 2017). Another example are ferritins, which have been recognized to be involved in the response to several kinds of oxidative stress (Chen et al. 2003; Tarrant et al. 2008) and related to cold resistance in the Antarctic krill (Clark et al. 2012). However, no significant differential expression between selective regimes was found in the present study for these families of genes, finding log_2-FC values that ranged from 0.57 to 1.52.

In conclusion, the comparative transcriptome analysis performed in this chapter revealed that genes related to diapause maintenance and termination were differentially expressed across rotifer diapausing eggs produced under two laboratory environments:

predictable vs unpredictable (Chapter 2). Genes related to embryo development reactivation were in general up-regulated in diapausing eggs from predictable environments, which is consistent with the higher hatching fraction of eggs produced under this selective regime. However, most of the genes related to diapause maintenance were also up-regulated in the predictable regime in both diapause conditions assayed. This study also extends the knowledge of the complex molecular and cellular events that take place during diapause. Some of the genes identified here are well known in other anhydrobiotic organisms and resistance forms, but several of them are new and should be further investigated to determine their involvement in desiccation and stress tolerance. Thus, further research is needed to achieve a better understanding of the present results and to furtherly elucidating the genes related to diapause in unpredictable environments. Given the future increase of environmental variability due to climate change (IPCC 2013), understanding the molecular mechanisms underlying the differential hatching patterns –like those found in the present and previous studies– is essential to understand how organisms cope with environmental unpredictability.

6

Final remarks and conclusions

A common objective in evolutionary ecology studies is to understand the adaptive significance and evolutionary potential of patterns of variation between and within natural populations. The starting point of evolutionary ecology as a discipline can be traced back to Charles Darwin's work (e.g., Egerton 2011). In his observations oriented at explaining the origin of species (1859), Darwin focused, above all, on the adaptation of organisms to their environment by natural selection. This tight and essential relationship between evolutionary theory and ecology through the concept of adaptation has become the core of the research programme of modern evolutionary ecology (sensu Lakatos 1970; McIntosh 1991). Evolutionary ecology lies in the interface of ecology and evolution and focuses in studying how organisms have evolved and adapted to their biotic and abiotic environment, and how current ecological processes interact with evolutionary history (e.g., Thompson 1998; Fox et al. 2001; Bijlsma and Loeschcke 2005).

Apart from natural selection, evolutionary ecology studies consider the other mechanisms of evolution (i.e., mutation, migration, random genetic drift, and mating preferences; e.g., Roughgarden 1979; Gillespie 1998; Lowe et al. 2017). However, understanding the interaction between these evolutionary mechanisms and the resulting phenotypic variation is not simple (Hendry 2016). Firstly, because trade-offs can arise between different phenotypic traits when a beneficial change in one trait is linked to a detrimental change in another (Stearns 1989; Flatt and Heyland 2012). Secondly, because mutation, inbreeding, drift, or migration can counterbalance the effect of other ecological selective factors, producing maladaptation (Crespi 2001; Olson-Manning et al. 2012). And thirdly, because both evolutionary and ecological processes can occur in short time-scales, interacting and modulating each other (Pelletier et al. 2009; Koch et al. 2014), thus producing the so-called "eco-evolutionary dynamics" (Fussmann et al. 2007; Pelletier et al. 2009; Schoener 2011; Hendry 2016). This notion changed the existing paradigm about evolution, which was considered to be only operating in long time-scales. Thereby, in order to put all these puzzle pieces together it is essential to understand the evolutionary trajectories of organism's phenotypic variation.

The advent of experimental evolution approaches –boosted by advances in high throughput techniques for genome sequencing and manipulation (Chapters 2 and 4)– has started allowing the

quantitative exploration of repeatability of evolutionary trajectories (Lobkovsky and Koonin 2012; Lang and Desai 2014; Orgogozo 2015; Bayley and Bataillon 2016; Fisher and Lang 2016), what is associated to the "replay the life tape" idea (Gould 1989; see Chapter 1). Experimental evolution provides an approach to study real-time adaptation in several (if not many) replicate populations. This research approach allows short-term evolutionary responses to be detected in simplified (usually laboratory) environments (Kassen 2002; Garland and Rose 2009; Kawecki et al. 2012; Kang et al. 2016), but with a high control of the factors that in nature could be interacting with the factor of interest (Matos et al. 2015; Fisher and Lang 2016). Gould's famous "tape of life" thought experiment has motivated many studies recreating evolutionary trajectories in the context of eco-evolutionary dynamics –i.e., interactions between ecology and evolution that play out on contemporary time scales– (Thompson 1998; Palkovacs and Hendry 2010; Hendry 2016). This field of research has contributed to the description of rapid evolution processes (e.g., Hairston et al. 2005; Fussmann et al. 2007; Declerck et al. 2015). In such context, rapid evolution has been defined as an evolutionary change occurring rapidly enough to have a measurable impact on a simultaneous ecological change (Hairston et al. 2005; Carroll et al. 2007). Experimental evolution has proven to be an efficient tool to determine if and how quickly traits diverge in response to selective

pressures (e.g., Lenski et al. 1991; Fussmann et al. 2007; Schrader et al. 2015; Declerck and Papakostas 2017). This thesis provides evidence of rapid and reproducible evolution in *Brachionus plicatilis* laboratory populations (Chapter 3). These rotifer laboratory populations underwent divergent evolution in the timing of sex and the hatching fraction of diapausing eggs in response to environmental unpredictability (Chapter 3), showing that –in agreement with previous studies– ecological change can lead to rapid evolutionary change (e.g., Thompson 1998; Yoshida et al. 2003; Hairston et al. 2005; Pelletier et al. 2009). In this experiment, independent replicate populations evolved the same phenotypic traits, what supports the repeatability of the experimental evolution outcomes, a question addressed in recent experimental evolution studies (e.g., Lobkovsky and Koonin 2012; Orgogozo 2015; Matos et al. 2015; Fisher and Lang 2016).

In the understanding of adaptive variation, it is not only important to study how selective pressures generate changes in phenotypic traits, but also the genetic architecture underlying these traits. Multiple next-generation sequencing (NGS) approaches have emerged over the last decade, in the so-called “omics era” (Van Straalen and Roelofs 2011; Tagu et al. 2014). These advanced technologies are providing unprecedented opportunities for addressing the molecular basis of adaptation in almost any organism, either if they are model organisms or not (Hudson 2008;

Twyford and Ennos 2012). This thesis has benefited of such technological improvements by applying both genomics (Chapter 4) and transcriptomics (Chapter 5). These technologies enable to find candidate genes under selection in different environments as well as genes that could be associated to specific phenotypic traits, such as life-history traits. In this thesis, candidate genes to be under selection in unpredictable environments and some other genes related to diapause have been identified (Chapters 4 and 5). Functional annotation is essential to make inferences about the functionality of the genes of interest, since it is what connects the nucleotide sequence to the biology of the organisms (Stein 2001). However, further inferences in this thesis cannot be drawn since, gene functional annotations for rotifer *B. plicatilis* –as for any other non-model species– are still scarce in the databases (Baric et al. 2016; Fuentes-Pardo and Ruzzante 2017). Nevertheless, the advances of NGS technologies (Chapter 1) are enabling the scientific community to expand the organisms of study –model and non-model– and to improve the functional information available in databases.

The analysis of the large amount of data generated by NGS technologies supposes a challenge for current bioinformatics (Mardis 2011; Elmer 2016; Esposito et al. 2016), what has resulted in an increased interest on the development of tools for mining ecological genomic data (Van Straalen and Roelofs 2011). This has

produced an increase of discovery-based studies with respect to hypothesis-based ones in this area of research (Coveney et al. 2016). Mostly, the research in this thesis is hypothesis-based (Chapters 3, 4 and 5), although some of the findings in Chapters 4 and 5 are also discovery-based (Dunn and Munro 2016). This issue should not be framed in terms of opposition (i.e., deduction versus induction, or hypothesis-based versus data-driven research) as both methods are necessary and can complement each other. Thus, despite the negative connotations associated to "descriptive", discovery-based studies (e.g., Kell and Oliver 2004; Casadevall and Fang 2008), they are necessary in the development of knowledge to provide a robust base for testable hypotheses in future hypothesis-based studies (Dunn and Munro 2016).

Understanding the process of adaptation to temporally varying environments is central in the current development of evolutionary ecology. The classic view of the environment in ecology regarded it as constant over time (Turchin 2001; from Malthus 1798). However nowadays, evolutionary ecology studies acknowledge that organisms and populations always live in fluctuating environments (Meyers and Bull 2002; Begon et al. 2006; see Chapter 1). Therefore, selective pressures change through time and environmental variability has to be taken into account to properly understand the diversity of evolved strategies. Moreover, the current scenario of increased environmental variability due to the

ongoing climate change (IPCC 2013) has attracted considerable attention on how species respond to changes in environmental variance (e.g., Botero et al. 2015; Lawson et al. 2015; Shama 2015; 2017). Coupling between ecological and evolutionary dynamics is expected under climate change because the latter will not only affect dispersion, local population dynamics and biotic interactions, but also the selective pressures shaping populations (Kinnison and Hairston 2007; Reed et al. 2011). One of the largest challenges in ecological studies aiming to understand the impacts of environmental variability is to characterize the patterns of variation of environmental fluctuations. However, this is not an easy task, which hinders testing evolutionary hypotheses in natural populations. Given that the predictability of environmental fluctuations can affect the fitness outcomes, it is necessary to perform an accurate quantification of environmental fluctuation (Franch-Gras et al. 2017a). To do this, it is mandatory (1) the availability of long-enough time series of relevant environmental factors (e.g., from historic records or remote-sensing data), and (2) the development of adequate metrics for measuring predictability (e.g., Fourier analysis, Colwell's metrics, Generalized Additive Models). These questions have been topic of recent research and have been successfully applied in some field studies (e.g., Sabo and Post 2008; Shine and Brown 2008; Sergio et al. 2011; Franch-Gras et al. 2017b). Interestingly, within the context of life-history trait

studies, the work by Franch-Gras et al. (2017b) showed evidence of local adaptation to environmental predictability in natural rotifer populations by using long-term satellite data on the water surface area of a series of ponds and lakes. The quantification of whether environmental fluctuations are prevalently predictable or unpredictable is not the only challenge that natural population studies have to face. Another difficulty is that confounding factors could occur in nature that can be misleadingly associated to environmental predictability (Hendry 2016). Experimental evolution can be a helpful and powerful tool to avoid these problems. This experimental approach allows for the production of close replicates, which is often complicated to attain in natural populations (Chapters 1 and 2; Matos et al. 2015; Fisher and Lang 2016). Although experimental evolution approaches have some recognized caveats (e.g., small population sizes or limited timescales of experiments; see Kawecki et al. 2012), these can be outweighed by the use of organisms with short generation times (Chapters 2 and 3). That is because this type of organisms provides many generations in experimentally manageable periods of time, what allows to observe short-term eco-evolutionary dynamics. This is the case of the rotifer *B. plicatilis*, used as a model organism to test evolutionary hypotheses in this thesis. In fact, monogonont rotifers have been recently recognized as suitable model organisms

in experimental evolution (revised in Declerck and Papakostas 2017).

Organisms living in temporally varying environments have developed strategies such as dispersal through time –i.e., "to travel in time"– to overpass unsuitable environmental periods (Venable and Lawlor 1980; Starrfelt and Kokko 2012b; Griffiths and Bonser 2013; Gerber 2018). This type of dispersal is frequently associated to the production of dormant stages, which can undergo dormancy during extended periods of time, working as a sort of "time machine" (Hairston 1998). Diapause is a type of dormancy considered to be adaptive when future conditions for survival or reproduction are unpredictable: producing active offspring ensures an evolutionary advantage in case the next season is favorable, whereas diapausing individuals have a high probability to survive through an unsuitable period and might reproduce in the future (Cohen 1966; Tuljapurkar 1990). Diapause is commonly associated with bet-hedging strategies (Hairston et al. 1985; Bradford and Roff 1993; García-Roger et al. 2017). In evolutionary ecology, bet hedging denotes risk-avoiding strategies that reduce temporal variation in fitness under environmental unpredictability (Cohen 1966; Seger and Brockmann 1987; Childs et al. 2010; Simons 2011; Ripa et al. 2010). This adaptive response can be achieved by using one of the two main modes of bet hedging: (1) conservative –i.e., when a single genotype produces a fixed, safe phenotype– or (2)

diversified –i.e., when a single genotype produces an array of different phenotypes in advance of future, uncertain conditions–. Bet-hedging theory was initially developed as a part of evolutionary biology dealing with the evolution of seed dormancy –i.e., the timing for leaving dormancy– in annual desert plants (Cohen 1966). Currently, bet-hedging theory is well defined, but there is a limited number of empirical studies (Simons 2011). In this thesis, a high level of empirical evidence on bet hedging in two life-history traits –the timing of sex and diapausing egg hatching fraction – has been achieved (Simons 2011; Chapter 3). It has been demonstrated that rotifer genotypes that hedge their bets in both the timing of entering and leaving diapause can be favored by selection in an unpredictably varying environment. Each trait is an instance of one of bet-hedging modes. On one hand, the earlier production of diapausing eggs –mediated by a prompter timing of sex– in rotifer genotypes from populations subjected to an unpredictable regime in the length of the growing season, with respect to those from a predictable one, is interpreted as an instance of conservative bet-hedging. On the other hand, the observation of intermediate diapausing egg hatching fractions in the populations subjected to the unpredictable regime provides support in favor of the existence of a diversified bet-hedging strategy. Remarkably, despite most of the studies on bet hedging typically focus on a single trait (Childs et al. 2010), this thesis shows that these strategies can act on a set of

traits, even if they have a similar effect to buffer the environmental variance.

This thesis' research shows the potential of combining different approaches –i.e., experimental evolution (Chapter 3), genomics (Chapter 4) and transcriptomics (Chapter 5)– to understand how organisms respond to unpredictable environments. The experimental evolution study on rotifer populations subjected to two contrasting regimes of environmental fluctuation (predictable vs unpredictable; Chapter 2) showed a rapid adaptive response to unpredictability (Chapter 3). Lower diapausing egg hatching fractions and earlier timing of sex were found in populations evolved under the unpredictable regime, suggesting the evolution of bet-hedging strategies in response to environmental unpredictability. Additionally, at the end of the evolution experiment, analysis of rotifer populations using NGS technologies identified genes putatively involved in adaptation to environmental unpredictability (Chapters 4 and 5). Nonetheless, further research is needed to fully understand how these traits interact and the role of bet hedging in the adaptation to environmental fluctuation. This will provide suitable information for predicting organisms' responses to environmental change (Lawson et al. 2015). Moreover, a better understanding of both *B. plicatilis* genome and transcriptome –besides the increase of genomic resource in

databases– is required for a complete elucidation of the genomic basis of adaptation to unpredictable environments.

Conclusions

The main conclusions derived from this thesis are enumerated below:

1. Populations of the rotifer *Brachionus plicatilis* rapidly evolved adaptive responses to environmental unpredictability under experimental evolution.

2. Diapause-related traits –the timing of sex and the hatching fraction of diapausing eggs– evolved divergently in laboratory populations subjected to two contrasting selective regimes of environmental fluctuation (predictable vs unpredictable).

3. The timing of sex was earlier in laboratory populations subjected to the unpredictable regime. This suggests a conservative, bet-hedging strategy that provides protection against unexpectedly short growing seasons.

4. Rotifer populations under the unpredictable regime evolved longer diapause (i.e., lower hatching fractions) than the populations under the predictable regime. This suggests a diversified bet-hedging strategy, promoting longer diapause

periods and favoring the survival of rotifer populations by its persistence in diapausing egg banks when the environment is unpredictable.

5. Asynchronous diapausing egg hatching was found in populations evolved under the predictable regime. This could be interpreted as a within-season bet hedging for this trait. Such within-season, risk-spreading strategy could evolve if the occurrence of successful growing seasons is predictable but there is some uncertainty with respect to their start.

6. Genotyping by sequencing (GBS) and subsequent bioinformatics analyses provided a large number (6,107) of high quality single nucleotide polymorphisms (SNPs).

7. Three SNPs –located in three different genes– showed a higher genetic differentiation between the selective regimes than expected by chance. Therefore, they are candidates to be under selection in unpredictable environments.

8. Genotype and phenotype analyses revealed four SNPs putatively associated to diapausing egg hatching fraction, and one SNP putatively associated to the timing of sex.

9. Parallel changes in allele frequencies of a number of candidate SNPs under selection were found in the six laboratory populations, independently of their selective regime. This

suggests a strong signal of adaptation to laboratory conditions during the selection experiment.

10. The negligible loss of genetic diversity, low values of inbreeding coefficient (F_{IS}), and little change in the fixation index (F_{ST}) between laboratory populations, suggest that genetic drift processes were not determinant in comparison to selection during the evolution experiment.

11. RNA-sequencing and subsequent bioinformatics analyses revealed a large number (3,068) of differentially expressed genes (DEGs) in the comparisons between selective regimes (predictable vs unpredictable), diapause conditions (non-forced vs forced) and their combinations.

12. Out of the 3,068 DEGs identified, 2,900 DEGs were found in the comparisons between the two contrasting selective regimes for both diapause conditions. This suggests that the selective regime is more important in driving differences in the transcriptome profile of diapausing eggs than the diapause condition assayed.

13. Most of the DEGs (2,815) were found to be up-regulated in the diapausing eggs produced under the predictable regime. These genes could be related to the reactivation of the embryo and hatching readiness, since diapausing eggs produced under the

predictable regime showed earlier and higher hatching fractions.

14. Genes related to the maintenance and termination of diapause were differentially expressed in *B. plicatilis* diapausing eggs produced under the two contrasting regimes of environmental fluctuation (predictable vs unpredictable).

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Appendix

Table A.1. Information of the ponds used for funding the laboratory populations in this thesis (obtained from Franch-Gras 2017).

Pond name	Acronym	Pond location	Estimated environmental predictability[a]
Petrola	PET	38°50'16.82"N, 1°33'49.22"W	1.00
Salobralejo	SAL	38°54'52.11"N, 1°28'6.95"W	1.00
Atalaya de los Ojicos	ATA	38°46'20.97"N, 1°25'49.12"W	0.75
Hoya Rasa	HYR	38°47'6.06"N, 1°25'37.56"W	0.66
Hoya Chica	HYC	38°49'46.22"N, 1°27'49.74"W	0.12
La Campana	CAM	38°51'29.06"N, 1°29'36.97"W	0.11
Hoya del Monte	HMT	38°50'44.87"N, 1°26'38.70"W	0.19
Hoya Yerba	HYB	38°46'46.02"N, 1°26'6.60"W	0.34
Hoya Turnera	HTU	38°46'31.19"N, 1°24'37.41"W	0.70

a. Estimated predictability calculated by COL_wd model (Franch-Gras et al. 2017a)

Table A.2. Summary of candidate SNPs under selection identified by BayeScan using three groups: (1) Origin population, (2) populations evolved under predictable selective regime, and (3) populations evolved under unpredictable selective regime. The three SNPs identified between two selective regimes are in bold. NA: no functionality found.

SNP_ID	*q*-value	F_{ST}	Gene association
S56713_7995	0.0000	0.3167	Transient receptor potential cation channel subfamily A member
S11659_1691	0.0000	0.2566	---NA---
S11659_1693	0.0000	0.2509	---NA---
S3012_36265	0.0000	0.2402	---NA---
S33929_139	0.0000	0.2340	---NA---
S33929_140	0.0000	0.2333	---NA---
S33929_141	0.0000	0.2330	---NA---
S16309_4100	0.0000	0.2251	cell division cycle
S16309_4101	0.0000	0.2243	cell division cycle
S16309_4103	0.0000	0.2242	cell division cycle

Table A.2. (continued)

SNP_ID	*q*-value	F_{ST}	Gene association
S1174_85150	0.0000	0.1965	Chloride intracellular channel exc-4
S13818_736	0.0000	0.1940	---NA---
S13818_738	0.0000	0.1831	---NA---
S2803_16180	0.0000	0.1775	CREB binding
S16698_16230	0.0000	0.1617	serine threonine- kinase tousled-like 2 isoform X4
S16698_16228	0.0000	0.1616	serine threonine- kinase tousled-like 2 isoform X4
S16698_16229	0.0000	0.16006	serine threonine- kinase tousled-like 2 isoform X4
S16698_16231	0.0000	0.1592	serine threonine- kinase tousled-like 2 isoform X4
S22481_5630	0.0000	0.1402	---NA---
S17011_30405	0.0000	0.1360	---NA---
S19655_8422	4.0008e-05	0.1352	homeobox prophet of Pit-1-like C:nucleus
S19655_8427	0.0000	0.1346	homeobox prophet of Pit-1-like C:nucleus

Table A.2. (continued)

SNP_ID	*q*-value	F_{ST}	Gene association
S19655_8423	0.0000	0.1342	homeobox prophet of Pit-1-like C:nucleus
S34894_4056	0.0000	0.1327	---NA---
S19655_8425	6.2513e-06	0.1324	homeobox prophet of Pit-1-like C:nucleus
S21617_6347	0.0000	0.1305	---NA---
S21617_6352	0.0000	0.1298	---NA---
S21617_6371	0.0000	0.1289	---NA---
S21617_6345	0.0000	0.1271	---NA---
S25654_622	0.0000	0.1197	photoreceptor-specific nuclear receptor-like.
S12176_10377	0.0000	0.1159	regulatory-associated of mTOR isoform X2
S78024_5745	0.0001	0.1069	Midasin
S32254_1877	9.7455e-05	0.1042	---NA---

Table A.2. (continued)

SNP_ID	*q*-value	F_{ST}	Gene association
S49622_8224	9.7455e-05	0.1023	---NA---
S49622_8242	0.0000	0.1008	---NA---
S49622_8228	4.0008e-05	0.1008	---NA---
S18850_7914	9.7455e-05	0.0998	transcription factor 7-like 2 isoform X8
S5177_8639	0.0033	0.0993	reverse partial 540 F:RNA-directed DNA polymerase activity
S5177_8631	0.0046	0.0992	reverse partial 540 F:RNA-directed DNA polymerase activity
S18850_7920	4.0008e-05	0.0988	transcription factor 7-like 2 isoform X8
S5177_8629	0.0027	0.0982	reverse partial 540 F:RNA-directed DNA polymerase activity
S5177_8630	0.0046	0.0973	reverse partial 540 F:RNA-directed DNA polymerase activity
S23450_12365	9.7455e-05	0.0933	myotubularin-related 13 isoform X5
S18850_7938	0.0001	0.0888	transcription factor 7-like 2 isoform X8
S10378_3420	0.0002	0.0878	pre-mRNA-splicing factor ATP-dependent RNA helicase DHX16

Table A.2. (continued)

SNP_ID	*q*-value	F_{ST}	Gene association
S54782_10127	0.0006	0.0856	adenylate cyclase type 2-like isoform X2
S47105_1467	0.015121	0.0851	---NA---
S22890_1785	0.0015407	0.0830	RNA-directed DNA polymerase from mobile element jockey
S18850_8016	0.0009	0.0819	transcription factor 7-like 2 isoform X8
S18850_7974	0.0004	0.0810	transcription factor 7-like 2 isoform X8
S2033_17976	0.0020	0.0807	---NA---
S10770_11251	0.0012	0.0807	partial F:zinc ion binding; F:metal ion binding
S12614_9116	0.0053	0.0766	sn1-specific diacylglycerol lipase beta
S49644_27362	0.0272	0.0764	---NA---
S40608_10312	0.0087	0.0762	---NA---
S40608_10343	0.0076	0.0756	---NA---
S49644_27363	0.0375	0.0754	---NA---

Table A.2. (continued)

SNP_ID	*q*-value	F_{ST}	Gene association
S9060_3689	0.0335	0.0748	RNA-binding single-stranded-interacting 3
S4644_2726	0.0060	0.0746	ribosomal S6 kinase alpha-1 isoform X1
S147401_780	0.0068	0.0731	sodium potassium-trasporting ATPase subunit alpha-1
S54782_10014	0.0100	0.0711	adenylate cyclase type 2-like isoform X2
S2597_97237	0.0131	0.0699	---NA---
S3404_8633	0.0171	0.0675	---NA---
S30218_6286	0.0116	0.0674	PREDICTED: uncharacterized protein LOC106168605
S3312_1846	0.0191	0.0665	Membrane-associated guanylate WW and PDZ domain-containing 2
S4239_2081	0.0251	0.0660	---NA---
S4239_2052	0.0211	0.0659	---NA---
S63942_1714	0.0314	0.0648	liprin-alpha-1-like isoform X3

Table A.2. (continued)

SNP_ID	*q*-value	F_{ST}	Gene association
S27662_4458	0.0399	0.0631	---NA---
S8852_22699	0.0231	0.0625	---NA---
S22745_2479	0.0355	0.0622	Condensin complex subunit
S8852_37112	0.0314	0.0618	---NA---
S5457_3425	0.0427	0.0604	---NA---
S11329_34316	0.0456	0.0575	isobutyryl- mitochondrial
S11329_32421	0.0484	0.0575	isobutyryl- mitochondrial

Table A.3. Allelic frequencies of each candidate SNPs under divergent selection between the origin and each of the laboratory populations. Pi: populations under the predictable one; Ui: populations under the unpredictable selective regime. The subindex i denotes replicate population within selective regime.

SNP	Origin	P_1	P_2	P_3	U_1	U_2	U_3
S1174_85150	0.760	1.000	1.000	1.000	1.000	1.000	1.000
S2033_17976	0.967	0.767	0.793	0.796	0.769	0.893	0.786
S2597_97237	0.694	0.850	0.917	0.839	0.667	0.190	0.614
S2803_16180	0.610	1.000	0.966	0.982	0.933	0.966	0.977
S3012_36265	0.677	1.000	1.000	1.000	1.000	1.000	1.000
S3312_1846	0.429	0.650	0.667	0.679	0.783	0.862	0.500
S3404_8633	0.985	1.000	0.950	1.000	1.000	1.000	1.000
S4239_2052	0.959	0.800	0.817	0.759	0.850	0.741	0.818
S4239_2081	0.959	0.800	0.817	0.759	0.850	0.741	0.818
S4644_2726	0.688	0.500	0.569	0.750	0.867	1.000	1.000
S5177_8629	0.925	1.000	1.000	1.000	1.000	1.000	1.000

Table A.3. (continued)

SNP	Origin	P_1	P_2	P_3	U_1	U_2	U_3
S5177_8630	0.925	1.000	1.000	1.000	1.000	1.000	1.000
S5177_8631	0.925	1.000	1.000	1.000	1.000	1.000	1.000
S5177_8639	0.925	1.000	1.000	1.000	1.000	1.000	1.000
S5457_3425	0.680	0.880	0.982	0.788	0.672	0.413	0.667
S8852_22699	0.816	0.783	0.700	0.875	0.350	0.500	0.386
S8852_37112	0.816	0.783	0.700	0.875	0.350	0.500	0.386
S9060_3689	0.924	0.914	0.724	0.857	1.000	1.000	1.000
S10378_3420	0.707	0.467	0.268	0.411	0.650	0.586	0.075
S10770_11251	0.528	0.793	0.692	0.731	0.929	0.981	0.667
S11329_32421	0.913	0.759	0.845	0.821	0.466	0.679	0.595
S11329_34316	0.903	0.786	0.860	0.833	0.448	0.648	0.528
S11659_1691	0.322	0.929	0.958	0.981	0.940	0.964	0.875
S11659_1693	0.330	0.929	0.958	0.981	0.940	0.964	0.875
S12176_10377	0.418	0.673	0.938	0.783	0.763	0.764	0.667

Table A.3. (continued).

SNP	Origin	P_1	P_2	P_3	U_1	U_2	U_3
S12614_9116	0.840	0.533	0.667	0.554	0.517	0.603	0.841
S13818_736	0.772	1.000	1.000	1.000	1.000	1.000	1.000
S13818_738	0.791	1.000	1.000	1.000	1.000	1.000	1.000
S13818_788	0.808	1.000	1.000	1.000	1.000	1.000	1.000
S16309_4100	0.864	0.212	0.173	0.286	0.308	0.295	0.312
S16309_4101	0.864	0.212	0.173	0.286	0.308	0.295	0.312
S16309_4103	0.864	0.212	0.173	0.286	0.308	0.295	0.312
S16698_16228	0.825	1.000	1.000	1.000	1.000	1.000	1.000
S16698_16229	0.825	1.000	1.000	1.000	1.000	1.000	1.000
S16698_16230	0.825	1.000	1.000	1.000	1.000	1.000	1.000
S16698_16231	0.825	1.000	1.000	1.000	1.000	1.000	1.000
S17011_30405	0.868	1.000	1.000	1.000	1.000	1.000	1.000
S18850_7914	0.378	0.519	0.704	0.375	0.917	0.853	0.850
S18850_7920	0.378	0.519	0.704	0.375	0.917	0.853	0.850

Table A.3. (continued)

SNP	Origin	P_1	P_2	P_3	U_1	U_2	U_3
S18850_7938	0.661	0.500	0.370	0.708	0.104	0.324	0.150
S18850_7974	0.555	0.700	0.767	0.554	0.950	0.931	0.886
S18850_8016	0.555	0.700	0.767	0.554	0.950	0.931	0.886
S19655_8422	0.873	1.000	1.000	1.000	1.000	1.000	1.000
S19655_8423	0.873	1.000	1.000	1.000	1.000	1.000	1.000
S19655_8425	0.873	1.000	1.000	1.000	1.000	1.000	1.000
S19655_8427	0.873	1.000	1.000	1.000	1.000	1.000	1.000
S21617_6345	0.817	0.964	1.000	1.000	1.000	1.000	1.000
S21617_6347	0.817	0.964	1.000	1.000	1.000	1.000	1.000
S21617_6352	0.817	0.964	1.000	1.000	1.000	1.000	1.000
S21617_6371	0.817	0.964	1.000	1.000	1.000	1.000	1.000
S22481_5630	0.865	0.591	0.450	0.237	0.382	0.591	0.433
S22745_2479	0.455	0.400	0.550	0.482	0.817	0.914	0.727
S22890_1785	0.391	0.722	0.741	0.568	0.818	0.574	0.719

Table A.3. (continued)

SNP	Origin	P_1	P_2	P_3	U_1	U_2	U_3
S23450_12365	0.405	0.600	0.800	0.750	0.817	0.672	0.705
S25654_622	0.699	0.982	0.926	1.000	0.933	0.920	0.975
S27662_4458	0.814	0.931	1.000	0.911	0.967	1.000	0.977
S30218_6286	0.532	0.533	0.650	0.607	0.850	1.000	0.795
S32254_1877	0.630	0.793	0.885	0.857	1.000	1.000	0.900
S33929_139	0.494	0.962	1.000	1.000	0.980	0.981	1.000
S33929_140	0.494	0.962	1.000	1.000	0.980	0.981	1.000
S33929_141	0.494	0.962	1.000	1.000	0.980	0.981	1.000
S34894_4056	0.739	1.000	0.967	0.982	1.000	1.000	0.955
S40608_10312	0.760	0.926	0.967	0.935	1.000	1.000	0.833
S40608_10343	0.760	0.926	0.967	0.935	1.000	1.000	0.833
S47105_1467	0.934	1.000	1.000	1.000	1.000	1.000	1.000
S49622_8224	0.694	0.914	0.933	0.946	0.967	0.981	0.932

Table A.3. (continued)

SNP	Origin	P_1	P_2	P_3	U_1	U_2	U_3
S49622_8228	0.694	0.914	0.933	0.946	0.967	0.981	0.932
S49622_8242	0.694	0.914	0.933	0.946	0.967	0.981	0.932
S49644_27362	0.840	0.827	0.854	0.900	1.000	1.000	1.000
S49644_27363	0.840	0.827	0.854	0.900	1.000	1.000	1.000
S54782_10014	0.750	0.917	0.933	0.911	1.000	0.948	0.909
S54782_10127	0.727	0.923	0.920	0.911	1.000	0.962	0.941
S56713_799	0.369	0.981	1.000	0.980	1.000	1.000	1.000
S63942_1714	0.722	0.707	0.185	0.500	0.397	0.667	0.400
S78024_5745	0.775	0.788	0.760	0.780	1.000	1.000	1.000
S147401_780	0.449	0.567	0.550	0.482	0.917	0.759	0.864

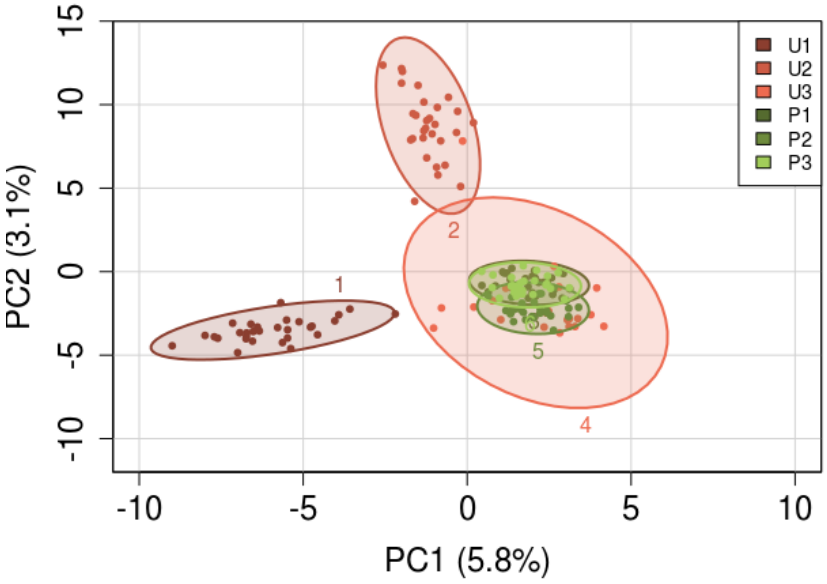
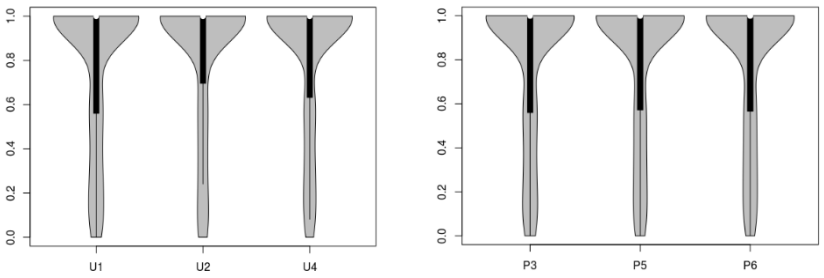


Figure A.1. Principal component analysis (PCA) plot for the 6,107 SNPs of *Brachionus plicatilis* clones from the six laboratory populations subjected to the two selective regimes (predictable vs unpredictable). Dots indicate the location of the genotype of each clone in the space defined by the first (PC1; 5.8 % variance explained) and second (PC2; 3.1 % variance explained) principal components. Ellipsoids are the 95% confidence interval the different populations. Color code: green, populations under predictable regime; red, populations under unpredictable regime.

(A)



(B)

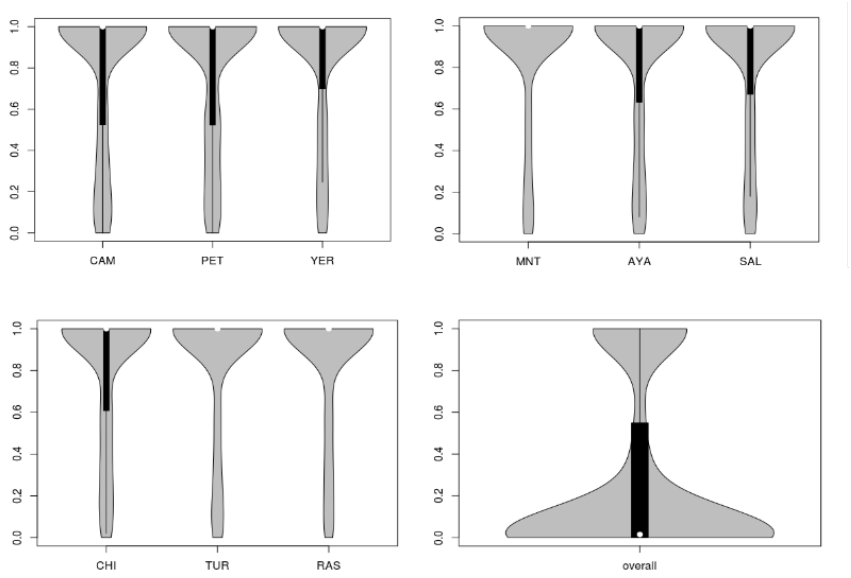


Figure A.2. Violin plots for results of Hardy-Weinberg (HWE) exact tests of the SNPs of each population. Tests for all populations (x-axis) and probability-values of the exact test (y-axis). (A) Laboratory populations. "U" represents populations under unpredictable regime and "P" represents populations under predictable regime. (B) Field populations.

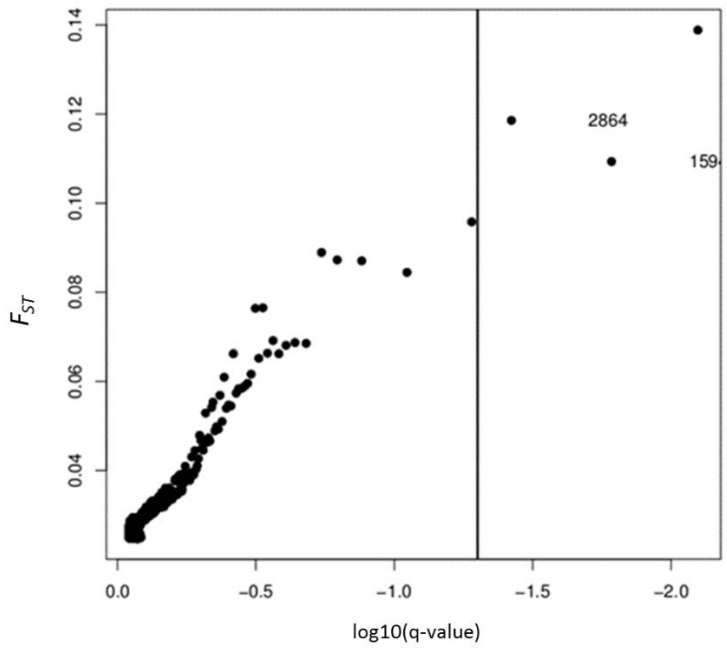
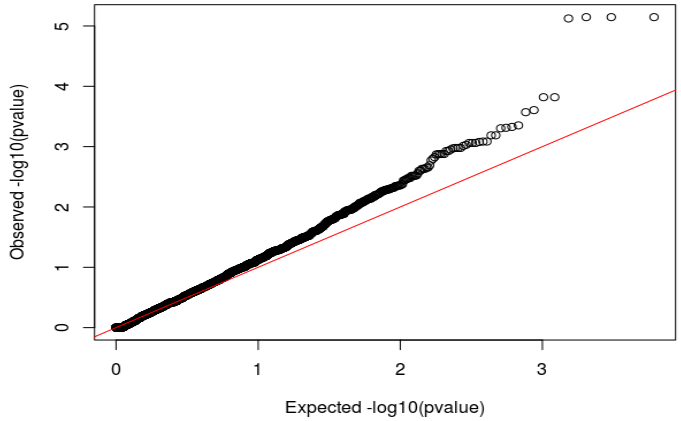


Figure A.3. Identification of outlier loci putatively under selection in *Brachionus plicatilis* populations using BayeScan analysis for the 6,107 genotyped SNPs. The marker-specific F_{ST} is plotted against the decision factor to determine selection in base-10 log scale $\log_{10}(q\text{-value})$ using a false discovery rate (FDR) of 0.05. The vertical line is the critical prior odds (PO) of 10 used to identify outlier markers. Markers on the right side of the vertical line are outliers. This analysis was performed using two groups: (1) populations subjected to the predictable selective regimen, and (2) populations subjected to the unpredictable one. Each dot represents a SNP. Red dots indicate SNPs identified as being putatively under selection between selective regimes

(A)



(B)

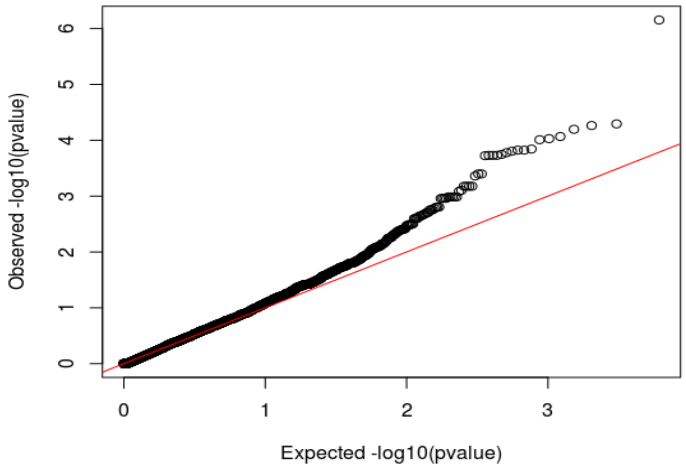


Figure A.4. Quantile-quantile plot of -log10 (*p*-value) for genotype-phenotype association anlayses. (A) Hatching fraction bioassay (λ = 1.27, SE = 0.0009) and (B) timing of sex bioassay (λ = 1.23, SE = 0.0014). Black dots represent the -log10 (*p*-value) of the entire study and the red line represents the expected values under no association.

Table A.3. Keywords used to find gene families related with diapause mainteinment and embryonic development.

Gene family	*Function*	*Keyword*	*References*
Diapause maintenance			
LEA proteins and trehalose			
Late Embryo Abundant (LEA)	Role in desiccation and stress tolerance	"lea"; "LEA"	Tunnacliffe et al. 2005; Goyal et al. 2005; Hand et al. 2007; Denekamp et al. 2009
Trehalose	Carbohydrate reserve and protection during water stress.	"tre"	Clegg 1965; Caprioli et al. 2004; Hand et al. 2007; Denekamp et al. 2009
Antioxidants			
Peroxidase activity	Combating oxidative stress	"perox" "stress" "oxi""	Denekamp et al. 2009; Clark et al. 2012
Catalase	Combating oxidative stress	"cata" "stress" "oxi"	Clark et al. 2012
Thioredoxin	Combating oxidative stress	"thio" "stress" "oxi"	Denekamp et al. 2009; Popovic et al. 2015

Glutathione-S-transferase	Combating oxidative stress	“glutat” “stress” “oxi”	Denekamp et al. 2009; Clark et al. 2012; Bryon et al. 2013
Ferritin	Combating oxidative stress	“ferrit” “artem” “stress” “oxi”	Clark et al. 2012; Popovic et al. 2015
Superoxide dismutase	Combating oxidative stress	“super” “dismu” “stress” “oxi”	Denekamp et al. 2009; Clark et al. 2012
Gamma-glutamyl transpepetidase	Combating oxidative stress	“gluta” “transp” “stress” “oxi”	Fan et al. 2013; Flabell 2017.
ABC transporters	Detoxifying and	“ABC” “stress” “oxi”	Bryon et al. 2013; Kang et al. 2015
Heat stress proteins			
hsp	Tolerance to thermal, cold, and osmotic stress. Also important in the initial embryo development	“hsp” “hsc” “heat” “stress” “chap”	Clegg et al. 2001; Denekamp et al. 2009; Clark et al. 2012; Fan et al. 2013; Popovic et al. 2015
Oxidoreductases			
Monooxygenases	Detoxification processes and	“cyt”; “P450”	Finkelstein et al. 2008; Clark

	degradation of xenobiotics.	“monoo” “stress”	et al. 2012; Bryon et al. 2013; Unal et al. 2013
Alcohol dehydrogenase	Enable the synthesis of cryoprotectants despite is also related with development	“alcohol” “deshy” “stress”	Clark et al. 2012; Tu et al. 2015; Kang et al. 2016b
Selenium binding proteins	Acts as antioxidant and can decrease lipid peroxidation	“seleni” “stress”	Morozova et al. 2003; Robich et al. 2007; Clark et al. 2012
Aquaporins			
Aquaporin	Water homeostasis, desiccation resistance and cold tolerance	“aqua”	Campbell et al. 2008; Philip et al. 2008; Denekamp et al. 2009; Clark et al. 2012; Drake et al. 2015
Lipid metabolism			
Fatty acid synthase	Lipid accumulation	“fatty”	Tan et al. 2016a
Fatty acid desaturase	Cold tolerance preserving membrane fluidity	“fatty” “des”	Clark et al. 2012; Sim and Delinger 2013;
Metyltranferase-like7	Involved in lipid droplet formation	“met”	Zehmer et al. 2008; Clark et al. 2012

Cathepsin protease	Yolk processing and degradation	“cathep”	Clark et al. 2012
Others; vitelline membrane	Components of egg shell	“vitel” “lipo” “lipid” “lipa”	Alekseev et al. 2012
Defense and protection			
Toll-like receptors	Innate immune system	“toll”	Roeder et al. 2004; Clark et al. 2012; Qi et al. 2015
Peptidase-C14		“pepti” “C14”	Clark et al. 2012
F-box and forkhead box	Protein degradation, signal transduction, cell cycle regulation. Expressed in diapause embryos of *Artemia*.	“box”	Kops et al. 2002; Lopes 2004; Qiu et al. 2007; Clark et al. 2012
WD-40	Expressed in *Artemia* diapause embryos.	“WD” “WD40”	Qiu et al. 2007; Clark et al. 2012
Lanthionine synthetase	Synthesis antimicrobial peptides	“lanthi”	Tanguy et al. 2004; Clark et al. 2012
Others			
Cholinesterase	Related with cryoprotection	“choline”	Bryon et al. 2013

Embryonic development			
Cytoskeleton			
Smoothelin	Specific protein for smooth muscle cells	“smoo”	Clark et al. 2012
Calponin	Actin-binding protein with a role in the regulation of cytoskeleton organization	“calpo”	Fu et al. 2009; Clark et al. 2012
Calmodulin	Calcium storage o binding calcium. Reactivation upon rehydration allowing hatch and development.	“calmo”	Alekseev et al. 2012; Clark et al. 2012; Ziv et al. 2017
Septin	Involved in cell signaling and components of cilia and flagella. Role in cytoskeletal restructuring.	“septin”	Mostowy and Cossart 2012; Hanson et al. 2013
Actin	Involved in hatching in *Culex pipiens*	“actin”	Kim et al. 2006
Morphological development			
Ependynim	Involved in cell proliferation and adhesion	“epend”	Clark et al. 2012
Countin	Involved in cell proliferation and adhesion	“count”	Clark et al. 2012

Notch	Not detected in resting eggs of *Brachionus*	"notch"	Clark et al. 2012
Innexins	Proteins that form gap junctions in invertebrates. Many of them have dynamic expression patterns during development in nematodes and are involved in neuronal development in the leech.	"innex"	Dykes and Macagno 2006; Clark et al. 2012; Simonsen et al. 2014
Vasotocin	Neuropeptide involved in neuronal development found in annelid worms and rotifers	"vasoto"	Tessmar-Raible et al. 2007; Clark et al. 2012
Osteonectin/BM-40	A calcium binding glycoprotein expressed in cells which undergoing morphogenesis, development and remodelling.	"osteo"	Motamed, 1999; Clark et al. 2012
Lipid metabolism			
Ecdysteroid-regulated protein	Essential for pupal diapause termination	"ecdy"	Denlinger et al. 2002; Ragland et al. 2011; Clark et al. 2012

Lipase family	Involved in fatty acid uptake, transport and metabolism	“lipa”	Denekamp et al. 2009; Poelchau et al. 2013
Lipooxigenase	Catalyze lipid conversion, involved in germination, stress-induced resistance, transition to flowering and defensive response in plants	“lipo”	Seltmann et al. 2010; Bañuelos et al. 2008; Vellosillo et al. 2007
Others			
Insulin growth factor	Down regulation induce diapause entrance	“insulin”	Sim and Delinger, 2008; Woll and Podrabsky 2017
Photoreceptor and pigments	Light photoreceptor which allow stimulation for hatching	“pigm” “carot” “rhodo” “photo” “opsin” “lux”	Kashiyama et al. 2010; Vanvlasselaer and De Meester 2010; Pinceel et al. 2013; Kim et al. 2015

* Orientative gene classification from Clark et al. 2012

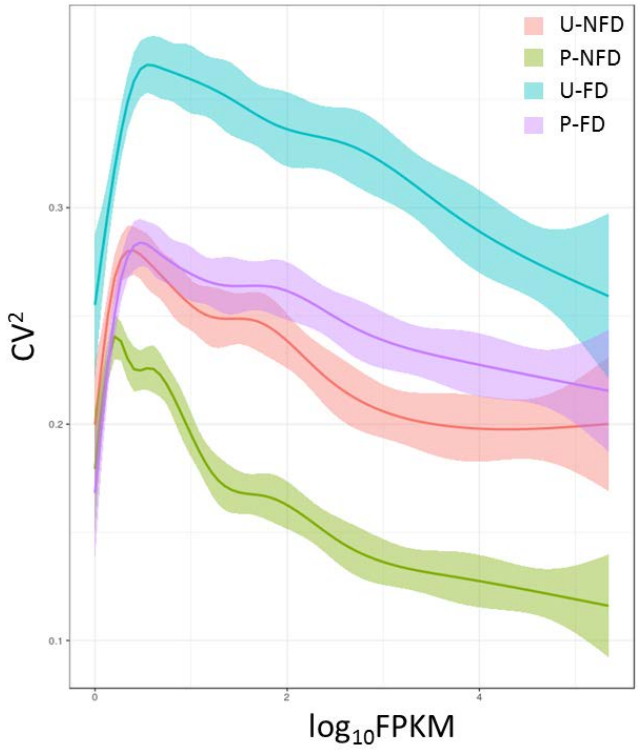


Figure A.5. RPKM density distribution plot (i.e., the squared coefficient of variation (CV^2) vs log_{10}FPKM) for each selective regime and diapause condition between the replicates.

(A)

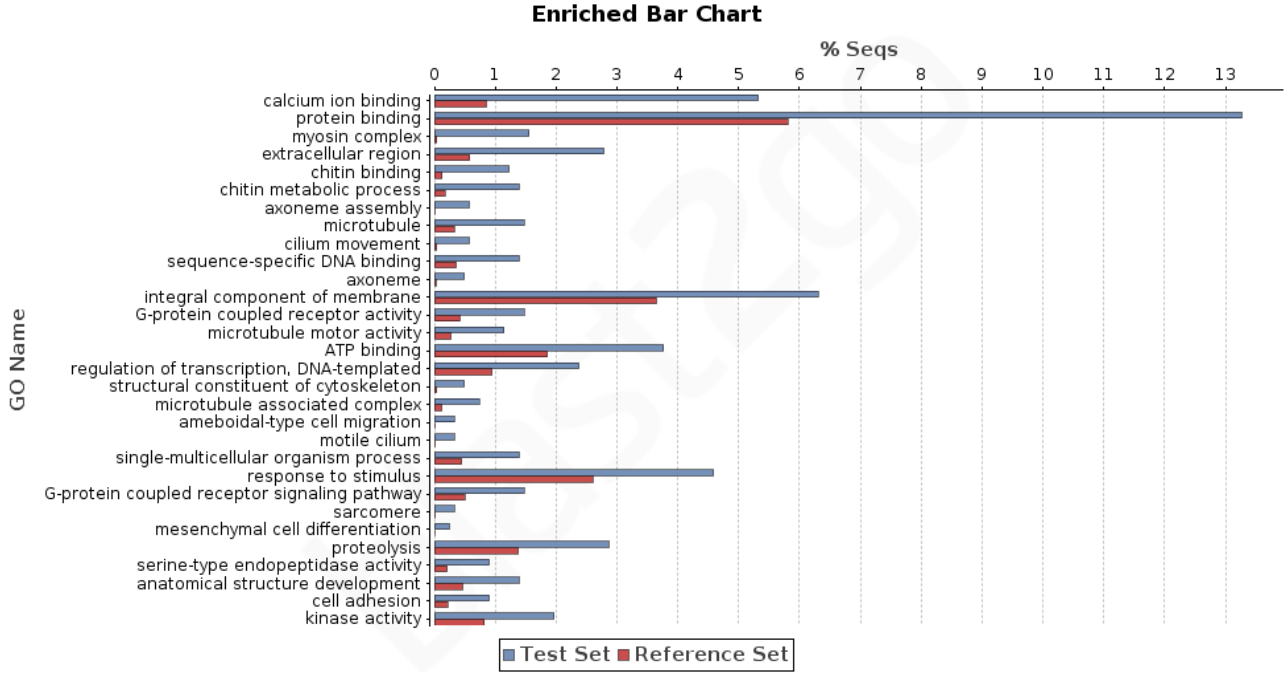


Figure A.6. GO enrichment analysis with the option reduced to most specific at FDR< 0.01. (A) U-NFD vs P-NFD comparison; (B) U-FD vs P-FD comparison.

(B)

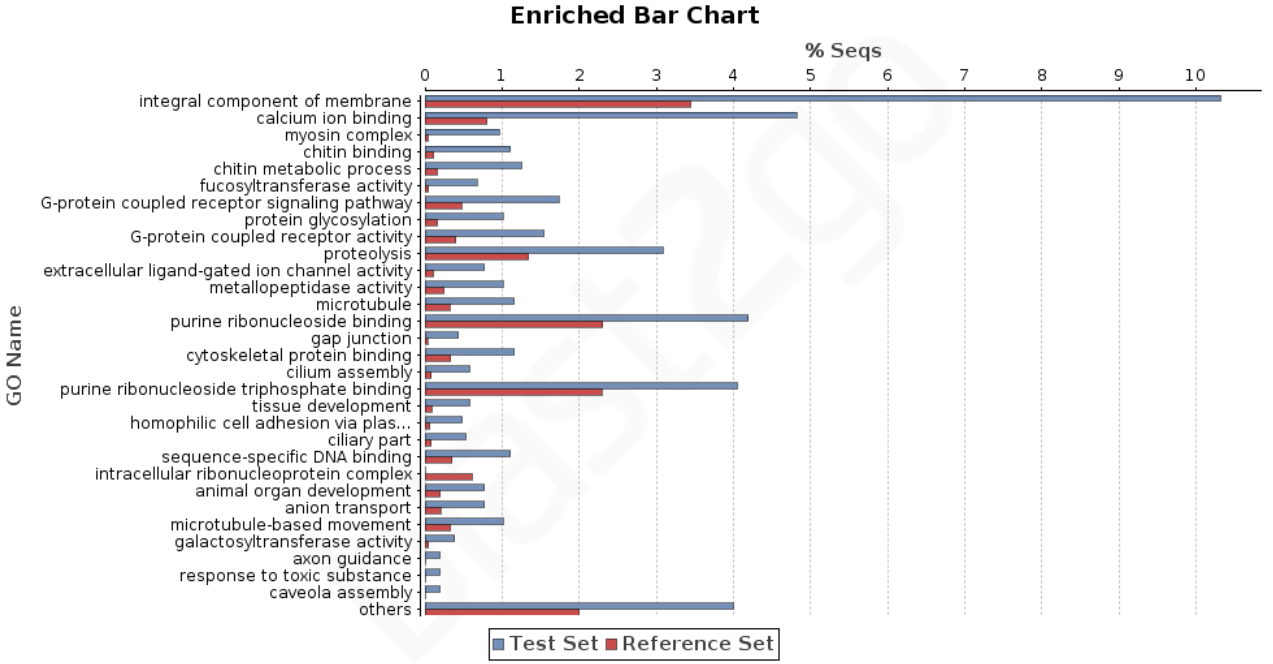


Figure A.6. (Continued).